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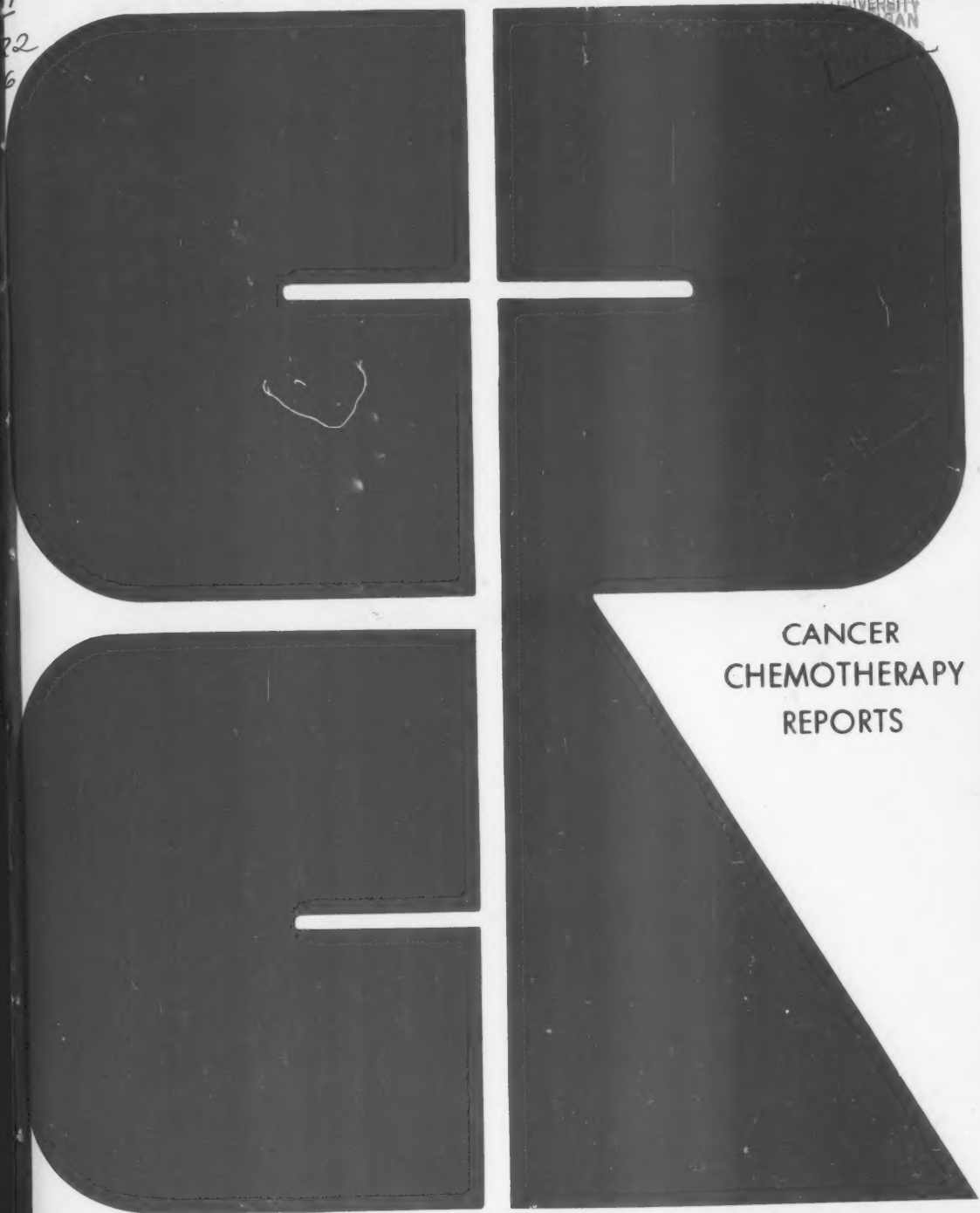

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Basic and Therapeutic Trial Results Obtained in the Spontaneous AK Leukemia (Lymphoma) Model—End of 1971 [1,2]

Howard E. Skipper, Frank M. Schabel, Jr., Mary W. Trader,
W. Russell Laster, Jr., Linda Simpson-Herren, and Harris H. Lloyd [3,4]

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SUMMARY

Basic and therapeutic trial results obtained in the spontaneous AK leukemia (lymphoma) model have been brought together for comparison with available information on the much used transplanted murine leukemia models and human leukemias and lymphomas.

The etiologic agent for "spontaneous" AK lymphoma is an RNA virus present at birth in AKR mice. Lymphoma cells first appear in the thymuses of animals at 5–>12 months of age. The time lapse between the first appearance of viable lymphoma cells in the thymus and clinical diagnosis (eg, with about 10^9 widely disseminated viable plus nonviable lymphoma cells in the host) is about 1 month. Thus, the overall doubling time of lymphoma cells before diagnosis is about 1 day. This estimate is compatible with the doubling time of relatively small numbers of first-passage lymphoma cells, assay data on the rate of repopulation of viable lymphoma cells after therapeutic reduction, and the median intermitotic time of dividing lymphoma cells (ie, 0.6 day).

In general, the cytokinetic parameters of advanced spontaneous AK lymphoma cell populations are more like those observed in advanced human leukemias than are those of early L1210 leukemia.

This paper presents assay data on the reduction in viable spontaneous AK lymphoma cells after treatment with a variety of agents, and the rate of cell repopulation after cessation of treatment.

Extensive therapeutic trial data indicate that cyclophosphamide is presently the most effective single agent against spontaneous AK lymphoma, with arabinosylcytosine or palmO-ara-C a close second.[5] Daunomycin, 5-fluorouracil, the nitrosoureas, vincristine, methotrexate, and dexamethasone provided moderate increases in host survival time. The combination of vincristine plus prednisone was a good remission inducer but the median survival time after cessation of treatment was shorter than that observed for cyclophosphamide or palmO-ara-C. The best responses observed to date with two-drug combinations appear better on several scores than the best that have been observed with single drugs. The best overall responses observed to date with two-drug combinations were with palmO-ara-C plus methyl-CCNU, cyclophosphamide plus methyl-CCNU, and palmO-ara-C plus cyclophosphamide. Some three- and four-drug combinations have provided better therapeutic responses than have been observed with single agents but not significantly better than those obtained with two-drug combinations.

Splenomegaly assays carried out immediately after cessation of treatment and 60 days and longer after cessation of treatment, suggest that eradication of all viable lymphoma cells is being achieved in some animals by combination chemotherapy; however, such animals eventually die of lymphoma, presumably as a result of the reinduction of a second lymphoma cell population. The requirements for permanent "cure" of spontaneous lymphoma in AKR mice include eradication of all viable lymphoma cells and prevention of reinduction.

Two major differences between early L1210 leukemia and clinically diagnosed spontaneous AK lymphoma are the degree of disease advancement at the time therapy is usually started (and associated cytokinetic differences) and the reinduction problem in AKR mice.

Spontaneous AK lymphoma is relatively more advanced at diagnosis than is acute leukemia in man (ie, with respect to nearness of the host to death), and it is presumed that the reinduction problem in AKR mice is more acute and more prevalent than in human neoplastic disease.

INTRODUCTION

I. General Background

Intensive study of transplanted murine leukemias has led to fairly broad cross-disciplinary understanding of the factors which underlie success and failure of therapy for such rapidly growing neoplasms (1–10). Strategies have been developed which result in the "cure" of transplanted leukemias by optimal single drug therapy (if the disease is not too advanced)

[5] Chemical nomenclature and NSC numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

or optimal combination chemotherapy (if the disease is quite advanced) (3,6,8–12).

Kinetic and therapeutic research has pointed out similarities and differences between rapidly growing murine leukemias and human leukemias and lymphomas (13–23). Although early transplanted murine leukemias (where the tumor burden does not exceed 10^5–10^6 leukemic cells) are useful screening tools and useful theoretic models for high growth fraction cancer cell populations, they are not ideal kinetic or therapeutic design models for human leukemias and lymphomas where 10^{11}–10^{12} cancer cells are present at diagnosis (13).[6]

For these reasons, much effort has been directed to the study of the cytokinetic behavior and response to therapy of spontaneous leukemias in animals.

In previous papers we have discussed our attempts to use spontaneous leukemia in the high leukemia stock of AKR mice as a model for human leukemias and lymphomas (24), and we have reported preliminary therapeutic trial results obtained with this system (25). Herein, we wish to (*a*) consider the biologic and cytokinetic behavior of this model in greater detail, (*b*) present quantitative data on the reduction of *viable*[7] lymphoma cells after therapy and the rate of repopulation of these cells, and (*c*) summarize and attempt to interpret additional therapeutic trial results obtained since earlier reports.

[6] The number of viable (or clonogenic) neoplastic cells present at diagnosis of human leukemias and lymphomas is not known, but it may be $<10^{11}$–10^{12}.

[7] After perhaps a century of basic and applied research aimed at cure of many types of cancer in man and animals, and varying degrees of success with surgical treatment, radiation therapy, and chemotherapy, we still have no really satisfactory single word to describe that particular cancer cell which so often defeats us; namely, a cell (or cells) which, if present after the end of treatment, has the immediate capacity to proliferate and give rise to some number of cancer cells which will kill the host. Such cells are variously referred to as cells with capacity for indefinite proliferation, cells with proliferative integrity, cancer stem cells, surviving tumor cells, viable tumor cells, clonogenic tumor cells, living tumor cells, etc. Herein we have used the word *viable* to refer to tumor cells which have the immediate capacity to act as the progenitor of a lethal cancer cell population. The fact that a given cell incorporates labeled thymidine, or excludes "vital stain," or appears to be "living" when viewed under the microscope is not good evidence of viability in the above sense.

II. Role of the Thymus in Leukemogenesis in the AKR Mouse

It is generally agreed that the vertically transmitted Gross virus (an RNA virus) is the etiologic agent for lymphoma in the AKR strain of mice (26,27) and that the lymphoma cells originate in the thymus (28).

As early as 1944, McEndy, Boon, and Furth demonstrated the role of the thymus in the development of leukemia in the AKR stock of mice (28). For example, total thymectomy performed on 1–2-month-old mice reduced the incidence of leukemia by about 87% (28).

In a recent report, Nakakuki et al (29) point out:

(1) "In AKR leukemia, the thymus is almost invariably the primary site of the disease and the organ most severely involved" (30,31).

(2) "Total thymectomy in young adulthood in this strain results in a marked decrease in the incidence of leukemias" (28,32,33).

(3) "Grafting of thymic tissue from AKR mice to thymectomized mice restores the susceptibility to leukemia development, and in many cases the tumors begin in the grafts themselves" (34). "Thymus grafts from histocompatible mice of low-leukemia strain have no such effect" (35,36).

(4) "In the AKR mice, the development of leukemia usually begins at 6 months of age." " . . . if the thymus merely provides local environment, noncellular or cellular, determining leukemogenesis, thymectomy at varying age would result in a similar effect on leukemia incidence."

In experiments designed to test the hypothesis that the thymus is the site of induction of neoplastic cells (rather than a tissue producing some humoral factor associated with leukemogenesis), these authors thymectomized AKR mice at 3, 35, 150, and 180 days of age and observed the effects on the incidence of lymphoma (table 1).

In keeping with the observations summarized in table 1, Nakakuki et al (29) report that "an histologic study on the earliest intrathymic

leukemia gave evidence that leukemia usually first appeared less than one month prior to clinical development of the disease."

We have observed that at diagnosis of spontaneous AK leukemia (by palpation of the lymphoid tissues, or determination of total white blood cell count, or both) the median thymus and spleen weights are 628 and 510 mg respectively; whereas, the normal thymus plus spleen weights usually total about 150–200 mg (24). The average number of lymphoma cells per milligram of thymus or spleen in AKR mice with spontaneous lymphoma is about 5.5×10^5. From these observations and from the knowledge that large numbers of leukemic cells are present in many other tissues, it is safe to estimate that at diagnosis of lymphoma AKR mice are bearing on the order of 10^9 leukemic cells (viable plus nonviable). If the earliest intrathymic leukemic cells usually first appear less than 1 month before clinical diagnosis, then the average overall doubling time of one (or a few) leukemic cells to 10^9 cells is of the order of 1 day or slightly less.

$$\left[\text{Ex. } 10^9 = 2^{30} \text{ (30 doublings)} \quad \frac{<30 \text{ days (from first histologically detectable intrathymic leukemia to diagnosis)}}{30 \text{ doublings}} = < 1 \text{ day (depending on the limits of histologic detection)}\right]$$

The above is not at all inconsistent with reliable doubling time measurements of spontaneous AK leukemia cells on first passage to syngeneic hosts (24). This doubling time value is 1 day and growth is essentially exponential over the range measured.

We emphasize the above in this introduction because we will have reason later to consider the doubling time of spontaneous AK leukemic cell populations during repopulation after therapeutic reduction (eg, after single doses or courses of drugs or between doses or courses). Also, we must face the question of "reinduction" of AK leukemia after therapy which eradicates all viable lymphoma cells and fails to affect the etiologic agent (25).

METHODS

The splenomegaly assay of Lin, Milley, and Bruce (37) has been used for determining both the presence or absence of viable lymphoma cells and the relative number of viable cells in tissues. When used in a quantitative manner this assay is a serial dilution method in which lymphoma cells are scored as viable when, on transplantation to young syngeneic recipients, they produce splenomegaly (or leukemic death) within 4 weeks. The relative number of viable lymphoma cells in a tissue suspension can be estimated from the successive fraction of animals (receiving tenfold dilutions) with enlarged spleens by application of statistical methods of Fisher and Yates (38) (tables 2 and 3).

When the assay was used in an all-or-none sense, the total thymus or spleen of a donor animal was suspended and implanted intraperitoneally (ip) into a single 2-month-old AKR mouse. Assay animals whose spleens weighed greater than 135 mg at the end of the assay period, or who died of leukemia in less than 4 weeks, were scored as positive. This cut-off spleen weight was based on a large number of concurrent control spleen weights of male and female AKR mice of 6–8 weeks of age (mean, 78 mg, with a standard deviation of 18 mg). During the present experiments, the extreme range of control spleen weights was 40–225 mg with only 1% greater than 135 mg.

The method used for clinical diagnosis of spontaneous AK lymphoma was routine weekly palpation of the spleen and lymph nodes, a procedure found to be satisfactory by Bruce (personal communication), Glynn (personal communication), and us. Figure 1 presents a summary of our historical experience, ie, host survival time after diagnosis of lymphoma. The data presented in figure 2 show no significant influence of age at diagnosis on median survival time after diagnosis.

EXPERIMENTAL STUDIES

I. Kinetics

A. Primary site and rate of growth of spontaneous AK lymphoma cells (before clinical diagnosis)

The thymectomy and histologic experiments already mentioned (29) suggest that the average time lapse between the induction of viable lymphoma cells in the thymus and the appearance of diagnosable lymphoma (about 10^9 widely disseminated lymphoma cells) may be of the order of 1 month.

The following experiments were carried out to shed more light on the presence of viable lymphoma cells in animals which had survived to specified ages but were not yet exhibiting clinically diagnosable disease. For example, if 50% of all AKR mice develop diagnosable lymphoma by 9 months of age and, say, 85% of a large sample of animals *which had survived to 9 months of age* are bearing no viable lymphoma cells in their thymuses or other tissues, then it may be concluded that about 43% ($0.50 \times 0.85 \times 100 = 42.5\%$) do not suffer a lethal carcinogenic event by 9 months of age. If sufficient information of this sort were available on AKR mice of different ages, it would be possible to relate the cumulative rate of induction of viable lymphoma cells to the rate of appearance of clinical disease and thus obtain an estimate of the average time lapse between induction and diagnosis.

With the above in mind, a group of 200 young AKR mice of known ages were set aside and groups of about 50 were killed for spleen and thymus assay at the ages of 3, 4, and 5 months. None of these animals had clinical evidence of lymphoma at the time of death for assay. Additional groups of 50 each (ages 6, 7, 8, 9, 10, 11, and 12 months) were selected from a large colony of AKR mice from which we routinely harvest animals as clinical lymphoma develops. In the age groups of 7–12 months some animals taken at random already had diagnosable disease, but many did not since clinical disease first appears at about 6 months of age and about 85% of the total beginning population are diagnosed with and die of lymphoma between the ages of 6 and 13 months.

Total thymus and spleen cells of the donor AKR mice (ages 3–12 months) were implanted ip into single young AKR mice for assay as described above. The results of this study are summarized in table 4. Only 7% of the animals bearing viable lymphoma cells showed evidence of viable cells in the spleen but not in the thymus, and these may have been false positives in the single animal assays. Forty-nine percent had positive assays for thymus but not for spleen and 44% had positive assays for both thymus and spleen, which would be expected if neoplastic cells originated in the thymus and metastasized to the spleen and other tissues before diagnosis. This observation is compatible with the fact that total thymectomy (up to 6 months of age) prevents development of spontaneous AK leukemia in a high percentage of animals.

A more detailed presentation and analysis of data summarized in table 4 may be found in table 5. When the fractions of animals that had survived to the ages indicated in table 4 (and still had no assayable lymphoma cells) are compared with extensive data on the cumulative incidence of clinical lymphoma and death in the total population (fig 2), some interesting relationships are observed. Namely, the incidence of undiagnosed but assayable lymphoma with age corrected for the cumulative incidence of diagnosable disease with age suggests the rate of induction of viable lymphoma cells (fig 3). It appears that the average time between induction of viable lymphoma cells in the thymus and the appearance of advanced and widely disseminated clinical leukemia (lymphoma) is slightly less than 1 month.

B. Doubling time of relatively small numbers of first-passage AK lymphoma cells, and the fraction which is viable on passage to syngeneic recipients

Extensive data on the relationship between the size of inoculum of first-passage AK lymphoma cells and the median lifespan of young syngeneic recipients are presented in figure 4. In these experiments counted numbers of lymphoma cells from many different donors were implanted intravenously (iv) into 6-week-old AKR mice. The data show that when lymphoma cells are taken from the thymuses of animals with advanced spontaneous disease (where

the total lymphoma cell population is approaching a plateau with respect to growth) and passed in relatively small numbers, the doubling time of these uncrowded lymphoma cells is about 1 day over the range of about 10^3–10^6 cells (about 1–10^4 viable cells). In addition, population growth is essentially exponential over this range.

These results also provide another basis for estimating the fraction of spontaneous lymphoma cells which have the capacity for unlimited proliferation (ie, are "viable" or "clonogenic"). The fractions of young AKR mice developing and dying of first-passage leukemia within 2 months after iv inoculation of counted numbers of cells are given in the legend to figure 4. The estimate that about 1% of the lymphoma cells are viable (on passage) is in agreement with results obtained using the splenomegaly assay (table 3). This is a median value; the extreme range is wide.

C. Cell cycle data and labeling indices in advanced spontaneous AK lymphoma cell populations

It has been shown that small, medium, and large AK lymphoma cells (fractionated by gradient centrifugation) all possess the capacity to cause a lethal lymphoma on passage to young AKR mice (39). The small lymphoma cells which have a low pulse labeling index (40) presumably are cells which were mostly in G_1 at the time when tritiated thymidine was administered. The large lymphoma cells which have a higher labeling index presumably represent cells which were in late S phase (and G_2) when a pulse dose of tritiated thymidine was administered. Table 6 summarizes pulse, 24-hour, and 48-hour labeling index data obtained for all cells in the thymuses and spleens of animals with advanced lymphoma. These labeling indices should be considered in the light of present evidence that, on the average, only about 1% of the lymphoma cells are viable on passage to syngeneic recipients.[8] The fact that a cell incorporated labeled thymidine is not necessarily good evidence that the cell has proliferative integrity.

Percent labeled mitosis curves which recently have been obtained for cells from the thymuses and spleens of animals with advanced spontaneous lymphoma are presented in figure 5. The procedures used have been described in earlier publications (41). The cell-cycle parameters given in figure 5 are in good agreement with those reported by Goncharova et al (42) for lymphomatous thymic and lymph node cells of AKR mice.

D. Period of in vivo exposure of spontaneous AK lymphoma cells to arabinosylcytosine versus reduction in viable lymphoma cells (ie, on the rate at which viable lymphoma cells initiate DNA replication)

Previous studies have provided evidence that arabinosylcytosine is S-phase specific and kills cells only during DNA duplication (10). Continuous exposure of rapidly growing L1210 leukemic cell populations (high labeling index, high fractional viability, and high growth fraction) to effective serum levels of arabinosylcytosine for 24 hours reduces the viable L1210 cell population by 99.999% (10).

The detailed results of quantitative splenomegaly assays on the thymuses of mice with spontaneous lymphoma after q3h (× 8) and q3h (× 16) regimens of arabinosylcytosine or a single 150 mg/kg dose of a depot form of arabinosylcytosine (palmO-ara-C) are given in table 7. Although considerable variation is observed from animal to animal, it appears that on the average about 97% of the *viable* spontaneous AK leukemic cells attempt DNA replication (and are killed by arabinosylcytosine) in 24 hours, about 99.9% in 38 hours, and about 99.99% in 48 hours.

The above information has both theoretical and practical implications. If, on the average, there are about 10^9 lymphoma cells in the AKR mouse when spontaneous lymphoma is diagnosed (of which about 10^7 are viable) and 24 hours' exposure to arabinosylcytosine reduces the viable lymphoma cells by only 2 decades (vs a 5-decade reduction in mice bearing 10^5 viable L1210 leukemia cells), the therapeutic implications are apparent. In this context it is pertinent that optimal regimens of arabinosylcytosine will "cure" animals bearing 10^5–10^6 first-passage AK lymphoma cells but fail to "cure" first-passage lymphoma if such therapy is delayed until this disease is more advanced.

[8] Obviously, some cell death may occur in the process of harvesting, suspension, and inoculation.

Additional data on the reduction of viable lymphoma cells after the administration of varying schedules of arabinosylcytosine and palmO-ara-C will be presented in the next section of this report, along with data on other types of drugs.

II. Distribution of Viable Lymphoma Cells at Diagnosis, Therapeutic Reduction, and the Rate of Repopulation After Therapy (or Between Doses or Courses)

In mice bearing spontaneous AK lymphoma, viable lymphoma cells are found in all tissues studied to date including blood, thymus, spleen, marrow, lymph nodes, liver, brain, lungs, kidneys, testes, and ovaries.

A. Distribution of viable lymphoma cells at diagnosis and at varying periods after a single tolerated dose of cyclophosphamide

The experiment summarized in table 8 provides quantitative data on the distribution of viable lymphoma cells at diagnosis and the effects of cyclophosphamide on such cells. Cytotoxic levels of the active form of cyclophosphamide are present in the serum of the mouse for only about 1 hour after a single dose of 150 mg/kg (43). Because the number of young AKR mice required for a single quantitative splenomegaly assay on a single tissue is rather large (approximately 24), the number of control and treated animals used in this experiment was small. Nevertheless, this pilot study indicates that a single 150 mg/kg dose of cyclophosphamide (0.5 LD10) rapidly renders a high fraction of the lymphoma cells nonviable in all tissues studied. However, by 8 days after treatment there had been notable repopulation and by 12 days there had been massive repopulation by progeny of surviving lymphoma cells. Also, it was noted that thymus and spleen weights regress to normal ranges in 4 days but then rather rapidly show evidence of lymphoma recurrence. A plot of the above results (fig 6) suggests that repopulation occurs at a doubling time of about 1 day. The rate of regression of thymus and spleen weights after therapy indicates the rapid "cell loss" resulting from cell death, lysis, and resorption.

Baseline data on thymus and spleen weights and the number of splenomegaly units per thymus in large numbers of untreated lymphoma-bearing AKR mice may be found in table 9.

B. Remission induction by vincristine plus prednisone and the rate of recurrence (based on viable lymphoma cells in the thymus, and thymus and spleen weights)

In an experiment somewhat similar to the one just described, groups of ten mice bearing clinically diagnosed lymphoma were treated with remission-inducing doses of vincristine plus prednisone and their thymuses assayed for viable lymphoma cells at 1 day and 11 days after cessation of treatment. Marked reduction and then rapid repopulation of viable cells was observed (table 10). A plot (fig 7) of the median values suggests that the doubling time of viable lymphoma cells, during repopulation after remission, is slightly less than 1 day.

C. Assays on the reduction of viable lymphoma cells in the thymus after treatment with various drugs

Lin, Milley, and Bruce (37) have reported the following reductions in viable spontaneous AK lymphoma cells in the thymus after therapy:

	% Reduction
Cyclophosphamide, 120 mg/kg (single dose)	99.999
BCNU, 10 mg/kg (single dose)	99.996
Vinblastine, 3 mg/kg/dose (q8h × 3)	99.4
Arabinosylcytosine (multiple doses over 24 hrs)	99.4

A summary of data we have obtained using the splenomegaly assay is presented in table 11. It is of interest to note that a 30-mg/kg single dose of arabinosylcytosine provided no measurable decrease in viable cells when assays were carried out 24 hours later. This would be anticipated because a 30-mg/kg dose of arabinosylcytosine provides a cytotoxic serum level for only about 3 hours, approximately 50% of the dividing cells are in S phase at a moment in time ($T_s/T_c=0.54$), and 21 hours is sufficient time for approximately one doubling of the surviving lymphoma cells. Significant cell kill after 5–10 daily doses of 30 mg/kg of arabinosylcytosine possibly suggests that a partial degree of temporary synchrony is being achieved.

III. Therapeutic Trials Against Spontaneous AK Leukemia (Lymphoma)

Data presented in figure 1 and tables 8 and 9

emphasize the advancement and heterogeneity of spontaneous AK lymphoma at diagnosis. It is clear that animals assigned at diagnosis to control and treatment groups cannot be as uniformly staged as is possible with transplanted murine leukemias. In our total experience from 1968 through 1971, 10% of untreated animals die within 4 days of diagnosis, 25% within 9 days, 50% within 16 days, 75% within 24 days, 90% within 31 days, and 99% within 55 days. This distribution may be expected to vary within groups of the size that can be used in reasonable therapeutic trials.

An indication of inherent variability of the spontaneous AK leukemia system, as we have employed it, may be seen in figure 8. This figure presents a plot of the distribution of the deviations in median survival time, after diagnosis, of 54 consecutive control groups of 35 untreated animals, each from their annual historical controls (fig 1). This analysis was carried out on groups of 35 untreated controls because groups of about this size have been used in therapeutic trials. The average deviation in median survival time of groups of 35 from the annual median has ranged from 1.3 to 2.5 days and the standard deviations have ranged from 2.0 to 3.8 days. In fact, an analysis of earlier control experience led to the decision to use about 30–40 animals in therapeutic trials. Our original expectation was that in groups of this size we could usually detect response to therapeutic regimens which had inherent capacity to increase survival time by as much as 50%, and we could rank therapeutic responses which differed by as much as 50% increase in lifespan (ILS). Subsequent experience appears to be compatible with this expectation.

When animals in the age range of 6–12 months are continually being harvested with lymphoma from a colony of 2000–3000 animals and each animal has a "different" spontaneous disease, the question of a perfect control is a difficult one. We have followed the practice of randomly assigning animals to control and treatment groups continually throughout the year and comparing the therapeutic trials with the total control experience for that year.

To minimize the data handling effort and to be able to follow the progress of each therapeutic trial, survival data are processed at specified intervals by a computer program which provides a printout of individual animal data and a plot of cumulative mortality for the group of treated animals and the group of control animals. Such charts are helpful in the analysis and interpretation of therapeutic trial results (figs 9–13).

Considerable thought has gone into the present tabulation of data obtained in numerous therapeutic trials against spontaneous AK lymphoma (tables 12–14). An attempt has been made to present the minimum amount of data necessary for comparison of the results obtained: (*a*) Dose, schedule, and day of last treatment are of obvious importance, as is toxicity data for normal mice for each therapeutic regimen used. (*b*) The percent ILS of treated groups over untreated control groups (percent ILS from day of diagnosis) is a useful value but can be misleading when the goal is "cure" and one is comparing therapeutic regimens of varying duration (eg, a daily dose schedule for, say, 15 days may kill neoplastic cells only about as fast as they are being replaced and thus provide significant increase in host survival time without ever approaching "cell cure"). (*c*) The fraction of animals dying during treatment is informative since it may suggest the fraction of animals in which a "useful" remission was not achieved or the fraction which did not receive the full planned therapeutic regimen, or in some instances it may suggest that a regimen, although not lethal for normal mice, was toxic for some animals with advanced lymphoma. Although not included herein, data are available on the thymus and spleen weights of all dying animals. These data suggest which animals die with advanced lymphoma and which die while in an apparent "remission." (*d*) The median lifespan of all animals in a treated group is a useful value but suffers from the same limitation as percent ILS. (*e*) The median lifespan of all animals after the end of treatment is pertinent in the context of knowledge that repopulation of viable lymphoma cells after therapy occurs at a doubling time of 1 day or slightly less (*see* figs 6 and 7 and compatible results in fig 4). The median survival time of those animals that survive

long enough to receive the intended therapy is of interest in the same context. (*f*) The percent of all animals in a treated group surviving 60 or 90 days or longer after the end of treatment is important in view of the evidence that the median time between the initial induction of viable lymphoma cells in the thymus and the diagnosis of clinical lymphoma is about 1 month or slightly less and the median time from clinical diagnosis to death is 16 days. We suspect that animals which survive for 60 days or longer after cessation of treatment (but die of lymphoma between 60 and 90 days or later) often represent animals in which all of the original lymphoma cells have been eradicated, and that their eventual death results from reinduction. More direct evidence on this point will be presented later.

Table 12 summarizes the results of 91 therapeutic trials with 16 single drugs. In some instances several schedules have been used. It should be emphasized that AKR mice bearing advanced lymphoma sometimes cannot tolerate dosages which are tolerated by nontumor-bearing mice or by mice bearing early transplanted murine leukemias. In our early experience we assumed that spontaneous AK leukemia did not respond to methotrexate because no increase in survival time was observed at dosages which regularly provided a good percent ILS in mice bearing early L1210 leukemia. It was then discovered that early first-passage AK leukemia responds to methotrexate as well as early L1210 leukemia. Subsequent studies showed that when the dosage of methotrexate was reduced, a moderate ILS of mice bearing advanced lymphoma was observed; however, methotrexate is a poor remission-inducing agent for this advanced disease.

Table 13 summarizes the results of 35 therapeutic trials with two-drug combinations. Our interest in the vincristine plus prednisone combination stems from the usefulness of this combination as a remission inducer in acute lymphocytic leukemia in children (44). Arabinosylcytosine plus thioguanine is potentiating against L1210 leukemia and is useful in the treatment of acute myelocytic leukemia (45,46). Combinations of cyclophosphamide plus a nitrosourea have been potentiating against transplanted murine leukemias and certain solid tumors (47,48), and combinations of arabinosylcytosine plus a nitrosourea or arabinosylcytosine plus cyclophosphamide have been very effective against advanced L1210 leukemia (11).

Table 14 summarizes the results of 15 therapeutic trials with combinations of three or more drugs. It should be apparent that the general approach in these trials was to obtain "remissions" with agents such as vincristine plus prednisone or palmO-ara-C and to follow as quickly as possible with other agents in an attempt to achieve "cell cure."

Although the results of combination chemotherapy trials have been listed in decreasing order of percent ILS of all animals from the day of diagnosis (tables 13 and 14), we hasten to emphasize that we do not consider this value to be an ideal single measure for comparing therapeutic response. Also, it should be clear that slight differences in percent ILS should not be considered significant in single therapeutic trials. At the present time we believe it is important to consider all of the end points listed in tables 12–14 in rating and interpreting therapeutic trial results. Obviously, our goal is to have as few animals as possible die during therapy and as many as possible survive without evidence of disease for as long as possible after cessation of therapy. As we work toward this goal we probably should consider the problem of "cure" in two dimensions: first, eradication of the viable tumor cells present at diagnosis, and secondly, the prevention of reinduction if the first goal can be achieved. From the practical standpoint it seems clear that the problem of reinduction of neoplastic diseases in man after surgical treatment, radiation therapy, or chemotherapy (solid tumors and leukemias and lymphomas) is not as immediate and prevalent as in the spontaneous AK leukemia model. With respect to eradication or nearness to eradication of viable lymphoma cells in AKR mice, we believe that median survival time after the end of treatment and the number of survivors 60 days after the end of treatment may be the most meaningful endpoints given in tables 12–14.

Assay evidence of "cell cure" in some animals

Quantitative splenomegaly assays have been carried out on the thymuses of mice that had

survived for more than 60 days after cessation of treatment with cyclophosphamide plus arabinosylcytosine (regimens for trials 133 and 134 in table 13). The results of these assays are summarized in table 15.

All-or-none assays have been carried out on a variety of tissues after therapy. In these assays the total organs (or a large fraction thereof) from treated animals were implanted ip into single young AKR mice and their spleens were weighed 28 days later. Again, spleen weights of greater than 135 mg in assay animals were scored as positive and spleen weights of 135 mg or less were scored as negative. The results of these experiments are summarized in table 16. The numbers of viable lymphoma cells in various tissues of untreated animals as determined by quantitative assay may be found in tables 2, 8, and 9.

IV. Prevention or Delay in the Development of Spontaneous AK Lymphoma

From what has been presented above, it seems that "cure" or very long control of spontaneous lymphoma in AKR mice, which carry an oncogenic RNA virus from birth, must entail chemotherapeutic eradication of all (or most) viable lymphoma cells, and that such therapy must be followed by secondary therapy which will prevent reinduction of a second lymphoma cell population.

The studies of Pollard and Sharon (49,50) using germfree AKR mice show that cyclophosphamide given once weekly prevents the appearance of diagnosable lymphoma over the age range of 5–13 months; however, when such treatment was stopped, diagnosable disease began to appear within 1–2 months. Holland et al (22) have shown that repeated immunization of AKR mice with neuraminidase-treated AK lymphoma cells between 2 and 4 months of age significantly decreased the rate of deaths from spontaneous AK lymphoma. We have observed that treatment of AKR mice with palmO-ara-C over the age range of 6–12 months delays the appearance of and death from spontaneous lymphoma. Data obtained in the above studies are summarized in table 17 along with results obtained after thymectomy.

DISCUSSION

I. Biologic and Kinetic Information

Sufficient data are now available on the spontaneous AK leukemia model to allow us to state some rather firm concepts and conclusions regarding:

(*a*) the etiologic agent,
(*b*) the primary site of the first lymphoma cells,
(*c*) the time lapse between the first appearance of lymphoma cells with immediate capacity for lethal proliferation and the appearance of clinically diagnosable lymphoma,
(*d*) the number of lymphoma cells in the host at clinical diagnosis and the fraction which is viable on passage to syngeneic recipients,
(*e*) the approximate overall doubling time of lymphoma cell populations from induction in the thymus to clinical diagnosis,
(*f*) the constancy of the doubling time of first-passage AK lymphoma cells over the range of one or a few viable cells to 10^4 or greater,
(*g*) the anatomic distribution of viable lymphoma cells at diagnosis,
(*h*) the median intermitotic time of dividing lymphoma cells in advanced disease and the median cell cycle phase parameters,
(*i*) the pulse and continuous labeling indices in lymphoma cell populations (regardless of individual cell viability),
(*j*) the capacity of small and large lymphoma cells to proliferate to a lethal number,
(*k*) the fractions of viable lymphoma cells which attempt DNA replication en route to division per unit time in advanced disease, and
(*l*) the median, distribution of, and range in survival time of AKR mice after diagnosis of spontaneous lymphoma.

Previously published and new data on the above points have been presented in preceding sections of the text and in tables 1–7 and figures 1–5. Briefly, the etiologic agent is an RNA

virus present at birth of AKR mice. The first viable (assayable) lymphoma cells appear in the thymus between 5 and 13 months of age. Such lymphoma cells proliferate rapidly and spread throughout the host before clinical diagnosis. At present our best estimate of the growth rate of "spontaneous" AK lymphoma cell populations, from induction of one or a few viable lymphoma cells to host death with greater than 10^9 viable plus nonviable lymphoma cells, is illustrated in figure 14.

Splenomegaly assays after therapy show the reduction in viable lymphoma cells in the thymus (tables 7–11) and other tissues (tables 8 and 16) and confirm the importance of schedule selection. Of equal importance is assay information indicating the rate of repopulation of viable lymphoma cells after therapeutic reduction (figs 6 and 7). Repopulation of viable lymphoma cells at a doubling time of about 0.7–1.0 day is compatible with best estimates of the doubling time of lymphoma cells before diagnosis and the more reliable doubling time of relatively small numbers of first-passage lymphoma cells, and the slightly shorter intermitotic time of dividing lymphoma cells in advanced spontaneous disease (ie, 0.6 day).

Extensive data on the reduction and rate of repopulation of normal femoral colony-forming cells in AKR mice (after chemotherapy), as well as quantitative data on long-passage AK lymphoma cells, may be found in the work of Bruce and associates (4,12,51,52).

II. Therapeutic Trials

Much of the above is pertinent to the design of therapeutic trials against spontaneous AK lymphoma and to the interpretation of therapeutic trial results. Such quantitative data also are required when one attempts to compare and relate therapeutic trial results obtained with this model to therapeutic trial results obtained with transplanted murine leukemias and human leukemias and lymphomas.

In our opinion, it is worthwhile to consider the therapeutic trial results obtained with the spontaneous AK leukemia model (tables 12–14) within the following context:

(1) Early first-passage AK leukemia (eg, 10^6 leukemia cells) is as sensitive to chemotherapy as L1210 leukemia (25). However, after "cell cure" of first-passage AK leukemia in 6-week-old AKR mice, spontaneous leukemia begins to appear at 5–6 months of age and such animals continue to die of spontaneous AK lymphoma at about the same rate as control animals (25). This is to say that "cell cure" of first-passage lymphoma in young AKR mice does not prevent induction of spontaneous lymphoma over the age range of 5–13 months.

(2) All animals used in therapeutic trials with the spontaneous lymphoma model are 6–12 months of age, have already been exposed to the etiologic agent for at least 6 months, and have suffered a carcinogenic event resulting in about 10^9 lymphoma cells at diagnosis. Such untreated animals will die from lymphoma within 16 days after diagnosis (median).

(3) If therapy fails to eradicate the lymphoma cells present at diagnosis, the surviving (viable) lymphoma cells repopulate rapidly after each dose or course or after cessation of treatment (eg, at a doubling time of about 0.7–1.0 day). If all but one or several viable lymphoma cells were eradicated in an animal, then recurrence from the original lymphoma cell population might be expected in about 30 days and death about 16 days later (on the average). On the other hand, if all of the original lymphoma cells were eradicated at the end of therapy and reinduction of a new population took place almost immediately, then recurrence might be expected 30 days later and death 16 days later, on the average (fig 13). It is obvious that such generalizations suffer because they rest on medians. They are offered here only as a framework for consideration of therapeutic trial results. In the simplest terms, if repopulation of lymphoma cells after therapy was at a constant doubling time of 1 day, then the following relationships would exist:

Reduction in viable lymphoma cells	Days to return to the original number
1 decade	3.32
2	6.64
3	9.96
4	13.3
5	16.6

Reduction in viable lymphoma cells	Days to return to the original number
6	19.9
7	23.3
8	26.6
9	29.9

Such approximate relationships are helpful in planning and attempting to interpret therapeutic trial results.

Of the single agents evaluated to date against spontaneous AK lymphoma (table 12), cyclophosphamide provided the best response according to several criteria, particularly median lifespan after cessation of treatment. When used optimally arabinosylcytosine and palmO-ara-C were nearly as effective as cyclophosphamide. When used alone the nitrosoureas provided only moderate increases in median host survival time. Daunomycin, 5-FU, vincristine, methotrexate, and dexamethasone provided only moderate increases in survival time.

The best responses observed to date with two-drug combinations (table 13) appear better on several scores than the best that have been observed with single agents (*see* trials 505, 666, 498, 502). It is of interest that the best overall responses observed with two-drug combinations were with palmO-ara-C plus methyl-CCNU, cyclophosphamide plus methyl-CCNU, and palmO-ara-C plus cyclophosphamide. Vincristine plus prednisone was a good remission inducer but the median survival time after cessation of treatment was shorter than that observed for cyclophosphamide or palmO-ara-C alone or other combinations listed in table 13.

Some of the three- and four-drug combinations (table 14) have provided better therapeutic responses than have been observed for single drugs, but not significantly better than the best responses obtained with two-drug combinations.

The survival time plots (figs 9–13) are of interest in view of the fact that spontaneous AK lymphoma is a very advanced and not ideally staged disease at initiation of treatment and the probability that reinduction follows cessation of treatment that eradicates the viable lymphoma cells.

The significance of 60-day or longer survivors after the end of treatment has been studied (table 15). The inability to detect viable lymphoma cells in the thymuses of 50% of such animals (and large numbers of viable lymphoma cells in only two of 12 animals) is convincing evidence that some of these survivors were rendered free of viable tumor cells at the end of treatment. It has been mentioned earlier that the doubling time of viable lymphoma cells during repopulation after remission induction by cyclophosphamide or prednisone plus vincristine is about 0.7–1.0 day.

Extensive qualitative splenomegaly assay data on a variety of tissues harvested 24 hours after cessation of therapy show residual viable cells in some instances but not in others (table 16). Generally, it appears that eradication of viable lymphoma cells is possible in all tissues studied when certain combinations are used. More data of this sort are needed.

At this point it seems propitious to raise a theoretical question on which we have no direct data: if "cell cure" is achieved in some 6–13-month-old AKR mice bearing advanced lymphoma (only to be followed by reinduction) would the fraction of such animals suffering reinduction per unit time be expected to be the same or higher or lower than the fraction of 6-month-old control AKR mice suffering their first induction of lymphoma cells (ie, per unit time)? After AKR mice reach the age of 6 months their rate of death from spontaneous lymphoma is about 20% per month. Analysis of all data on 60- versus 90-day survivors after the end of treatment (regardless of treatment, [*see* tables 12–14]) indicates that of 243 animals which survived 60 days, only 97 (40%) were alive 90 days after cessation of therapy. Thus, one must suspect that either all animals surviving 60 days after the end of treatment do not represent "cell cures," or that the fraction suffering reinduction per unit time is considerably higher than the fraction having original induction of lymphoma per unit time.

Some differences between experimental leukemias and human leukemias are listed in table 18. Major differences between L1210 leukemia and spontaneous AK leukemia are (*a*) the degree of advancement at the time the animals

are usually entered into therapeutic trials, (*b*) other parameters often associated with early versus advanced neoplastic diseases, and (*c*) the fact that if all the viable lymphoma cells are eradicated in AKR mice, reinduction may be expected if the etiologic agent is not suppressed or eradicated.

With respect to relationships between spontaneous AK lymphoma and human leukemias and lymphomas, the AKR mouse is closer to death at diagnosis (time-wise), and the reinduction problem appears to be a more immediate and prevalent problem than is known with any human neoplastic disease. The kinetic parameters of advanced spontaneous AK lymphoma are more like those of acute leukemias in man than those of early transplanted murine leukemias.

Regarding response to different classes of drugs, spontaneous AK leukemia mimics a variety of human leukemias and lymphomas in certain respects (eg, remission induction by vincristine plus prednisone as in acute lymphocytic leukemia, remission induction by arabinosylcytosine as in acute myelocytic leukemia, good response to cyclophosphamide perhaps as in Burkitt's lymphoma and Hodgkin's disease and other lymphomas), but it probably is not a perfect model for any one human neoplastic disease. Host differences such as are listed at the bottom of table 18, of course, must be taken into consideration when relating and translating therapeutic trial regimens and results.

Finally, future efforts to provide lifetime control or "cure" of spontaneous AK lymphoma must include measures for eradicating all viable lymphoma cells in as high a percentage as possible and then preventing reinduction.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Arabinosylcytosine: NSC-63878; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C; cytosine arabinoside
BCNU: NSC-409962; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
CCNU: NSC-79037; urea, 1-(2-chloroethyl)-1-nitroso-
Cyclophosphamide: NSC-26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Daunomycin: NSC-82151
Dexamethasone: NSC-34521
5-Fluorouracil: NSC-19893; 5-FU
Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Methyl-CCNU: NSC-95441; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-
Nitrogen mustard: NSC-762; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride
PalmO-ara-C: NSC-135962; 1-β-D-arabinofuranosylcytosine, 5′-palmitate
Prednisone: NSC-10023; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
L-Sarcolysin: NSC-8806; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-
6-Thioguanine: NSC-752; purine-6-thiol, 2-amino-
Vincristine: NSC-67574; leurocristine, sulfate (1:1)

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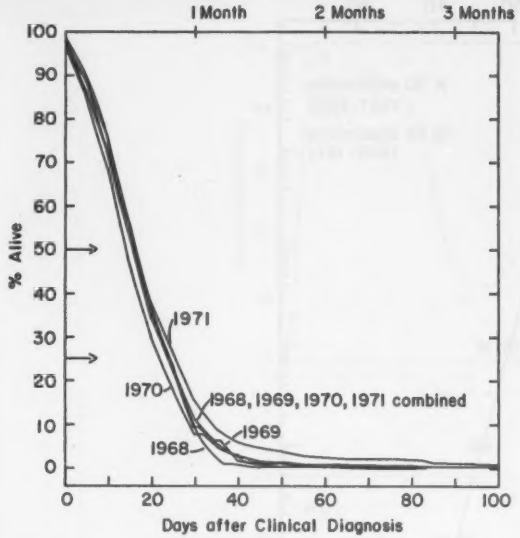

FIGURE 1.—Survival time of untreated control animals after clinical diagnosis of spontaneous AK lymphoma.

	Lifespan (days)		No. surviving/total treated	
	Median	Last 25%	> 60 days	> 90 days
1968*	17	24	2/497	1/497
1969*	16	23	0/256	0/256
1970†	14	22	1/631	0/631
1971†	17	24	10/527	6/527
Total experience	16	23	13/1911	7/1911

*Diagnosed by palpation of spleen, nodes, and thymus, plus the requirement of a wbc count $> 25{,}000$ cells/mm³.

† Diagnosis by palpation only.

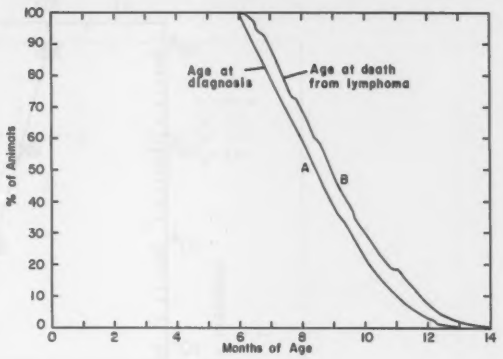

FIGURE 2.—Age at diagnosis of spontaneous lymphoma vs age at death from lymphoma. A = Age at diagnosis of lymphoma (1970; 5, 146 animals). B = Age at death from lymphoma (1970; 636 untreated controls). Only those animals developing and dying of lymphoma have been included in this plot.

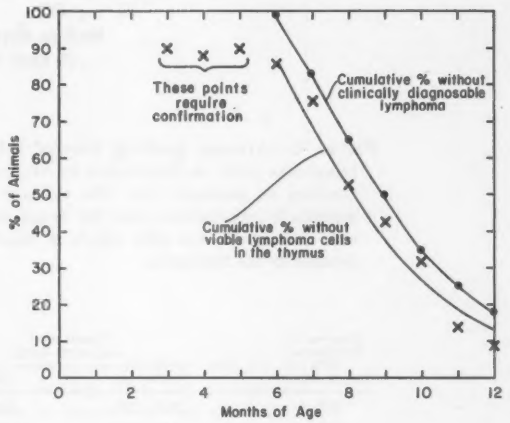
These points require confirmation
Cumulative % without clinically diagnosable lymphoma
Cumulative % without viable lymphoma cells in the thymus
% of Animals
Months of Age

FIGURE 3.—Estimation of the cumulative rate of appearance of viable lymphoma cells in the thymus vs the observed cumulative rate of appearance of clinically diagnosable lymphoma.

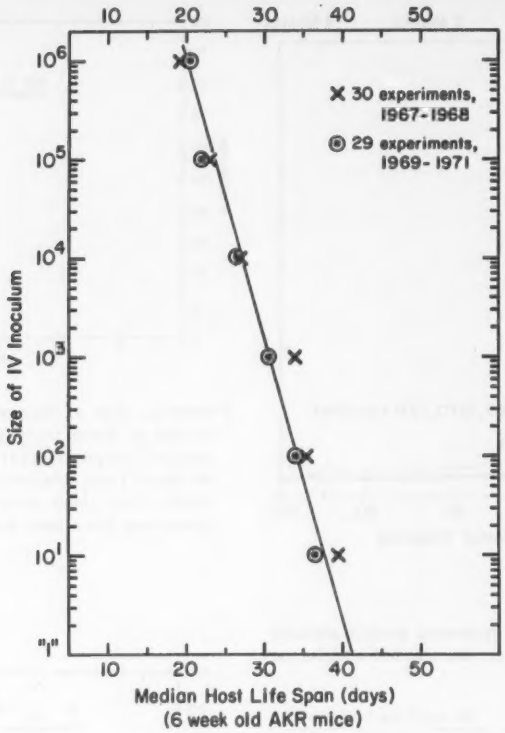

FIGURE 4.—Average doubling time of relatively small numbers of first-passage AK lymphoma cells as determined by iv titration in young AKR mice (and the viable fraction on passage). (*a*) The average doubling time is 1 day and the growth is essentially exponential over the range studied. (*b*) The median fraction of spontaneous AK lymphoma cells which is viable on passage to syngeneic recipients is indicated by the following:

Number of lymphoma cells implanted iv	Fraction of recipients dying		Percent of total group dying of lymphoma within 2 months	Median survival time* (days)
	1967–1968	1969–1971		
10^6	293/298	500/502	99	20
10^5	243/248	266/266	99	23
10^4	229/255	261/265	94	27
10^3	144/203	211/266	76	31
10^2	72/179	41/92	42†	34
10^1	10/87	9/80	11	37

*Read from line of best fit.

†On the average, 42% of animals receiving 100 cells developed and died of passage lymphoma. Thus, on the average, 100 cells contained about 1 viable cell as measured by the ability to induce lethal lymphoma in young syngeneic recipients. This estimate of about 1% viability on passage is in agreement with results obtained using the splenomegaly assay (table 3).

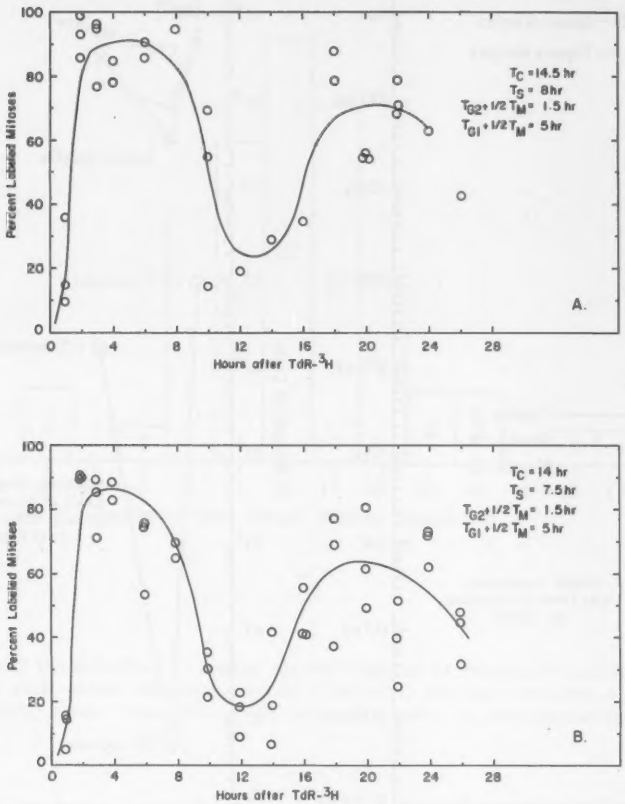
Percent Labeled Mitoses
Hours after TdR-^{3}H
T_C = 14.5 hr
T_S = 8 hr
T_{G2} + 1/2 T_M = 1.5 hr
T_{G1} + 1/2 T_M = 5 hr
A.
T_C = 14 hr
T_S = 7.5 hr
T_{G2} + 1/2 T_M = 1.5 hr
T_{G1} + 1/2 T_M = 5 hr
B.

FIGURE 5.—Percent labeled mitosis curves on dividing cells in the thymuses and spleens of animals bearing advanced spontaneous lymphoma. A. Cell cycle of all cells in thymus. B. Cell cycle of all cells in spleen. Values reported by Goncharova et al (42): T_C = 15 hrs, T_S = 8 hrs, T_{G_2} = 2.2 hrs, T_{G_1} = 4.8 hrs.

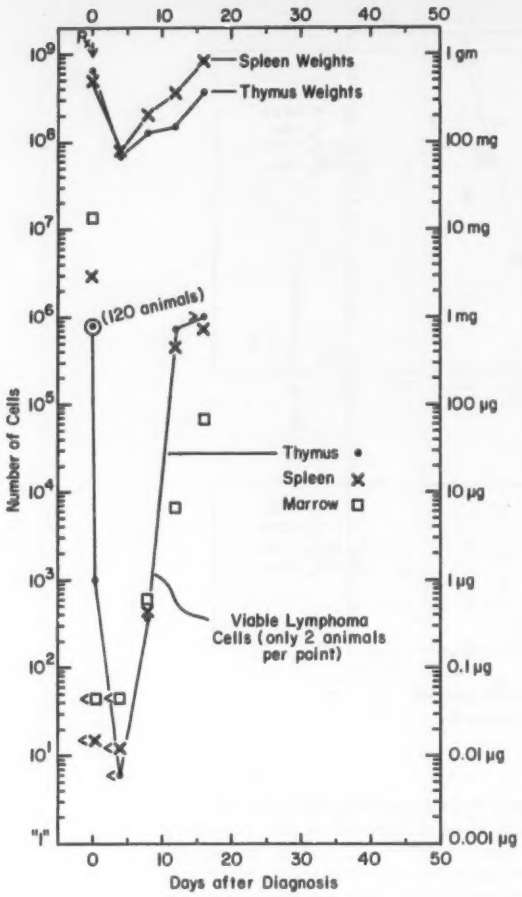

FIGURE 6.—Remission induction by a single dose of cyclophosphamide, and rate of repopulation of viable lymphoma cells in the thymus, spleen, and marrow.

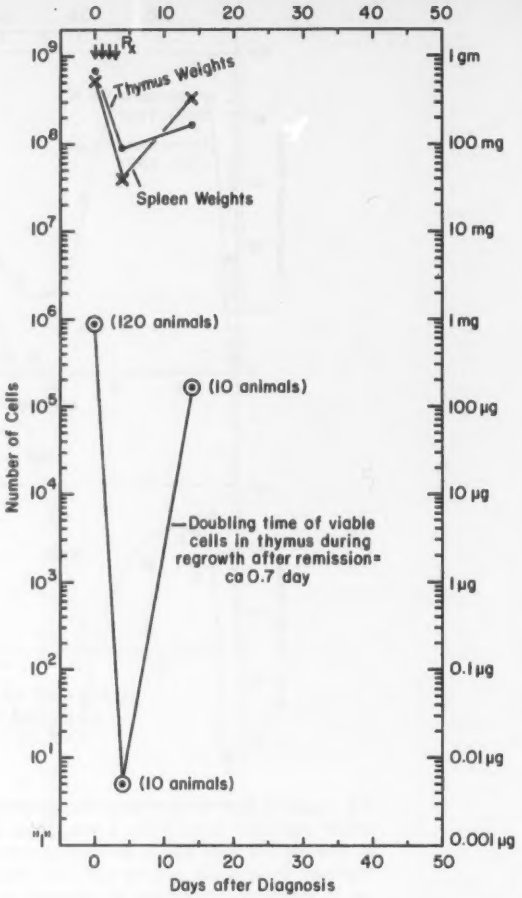

FIGURE 7.—Remission induction in spontaneous AK lymphoma by vincristine plus prednisone, and rate of repopulation of viable lymphoma cells (median values).

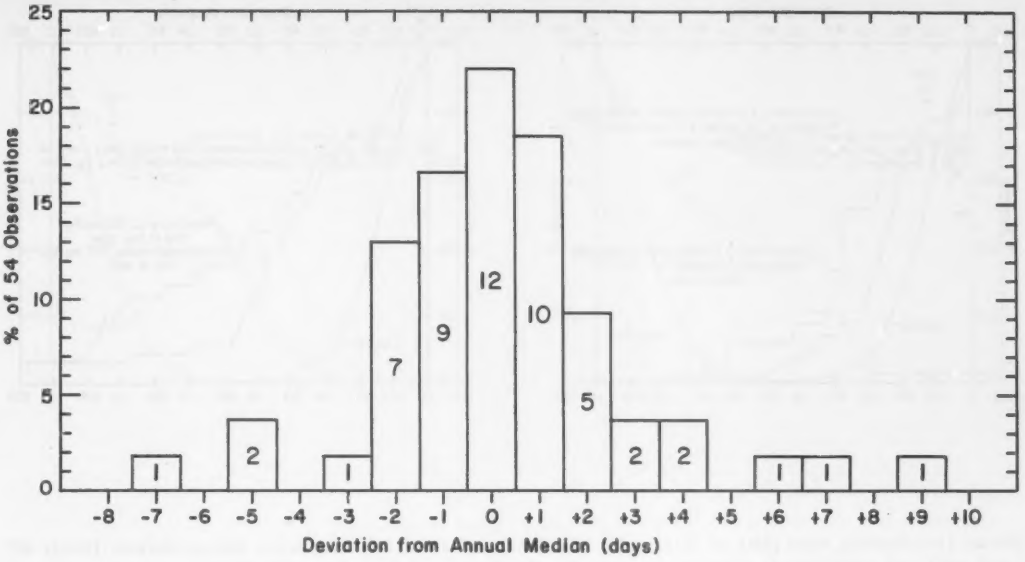

FIGURE 8.—Distribution of the deviations in median survival time of 54 consecutive control groups of 35 untreated animals from their annual historical controls (1968–1971). Standard deviation = 2.7 days; number of groups indicated; slightly greater than 80% of the values fall within ±1 standard deviation.

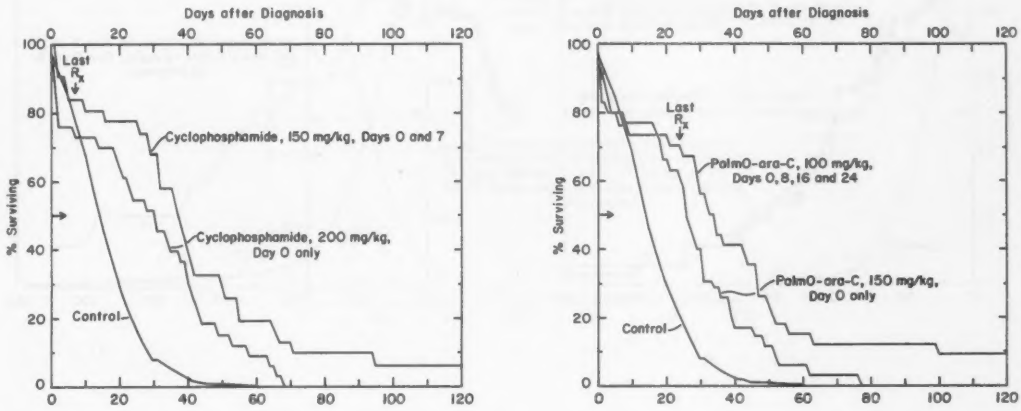

FIGURE 9.—Survival time plots of therapeutic trial results obtained with cyclophosphamide (trials 399 and 410) and palmO-ara-C alone (trials 393 and 509) (*see* table 12).

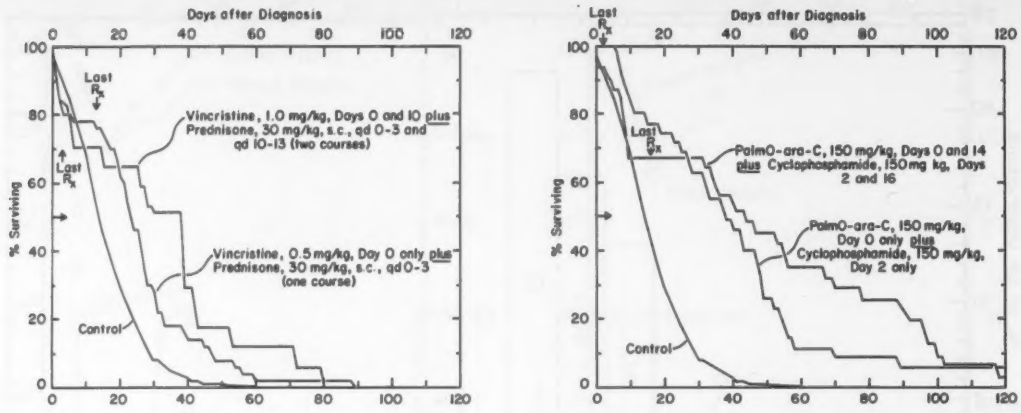
Days after Diagnosis
% Surviving
Last Rx
Vincristine, 1.0 mg/kg, Days 0 and 10 plus Prednisone, 30 mg/kg, s.c., qd 0-3 and qd 10-13 (two courses)
Vincristine, 0.5 mg/kg, Day 0 only plus Prednisone, 30 mg/kg, s.c., qd 0-3 (one course)
Control
PalmO-ara-C, 150 mg/kg, Days 0 and 14 plus Cyclophosphamide, 150 mg kg, Days 2 and 16
PalmO-ara-C, 150 mg/kg, Day 0 only plus Cyclophosphamide, 150 mg/kg, Day 2 only

FIGURE 10.—Survival time plots of therapeutic trial results obtained with vincristine plus prednisone (trials 463 and 468) and palmO-ara-C plus cyclophosphamide (trials 498 and 539) (*see* table 13).

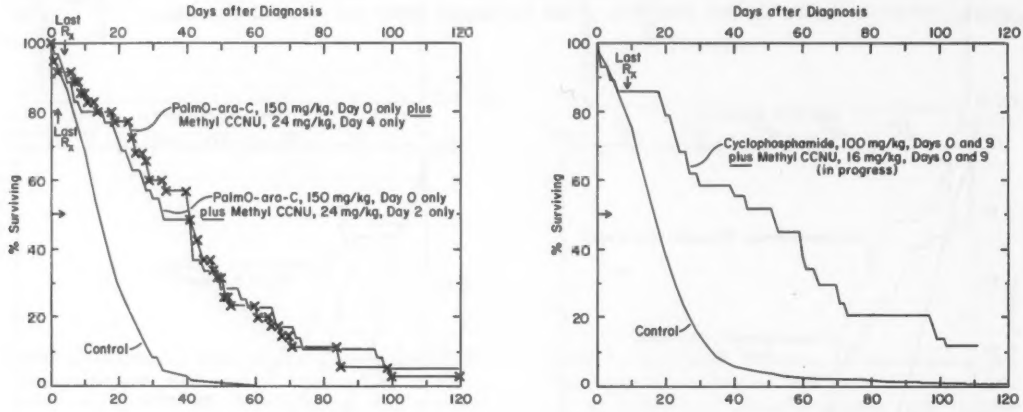
Days after Diagnosis
% Surviving
Last Rx
PalmO-ara-C, 150 mg/kg, Day 0 only plus Methyl CCNU, 24 mg/kg, Day 4 only
PalmO-ara-C, 150 mg/kg, Day 0 only plus Methyl CCNU, 24 mg/kg, Day 2 only
Control
Cyclophosphamide, 100 mg/kg, Days 0 and 9 plus Methyl CCNU, 16 mg/kg, Days 0 and 9 (in progress)

FIGURE 11.—Survival time plots of therapeutic trial results obtained with palmO-ara-C plus methyl-CCNU (trials 503 and 505) and cyclophosphamide plus methyl-CCNU (trial 666) (*see* table 13).

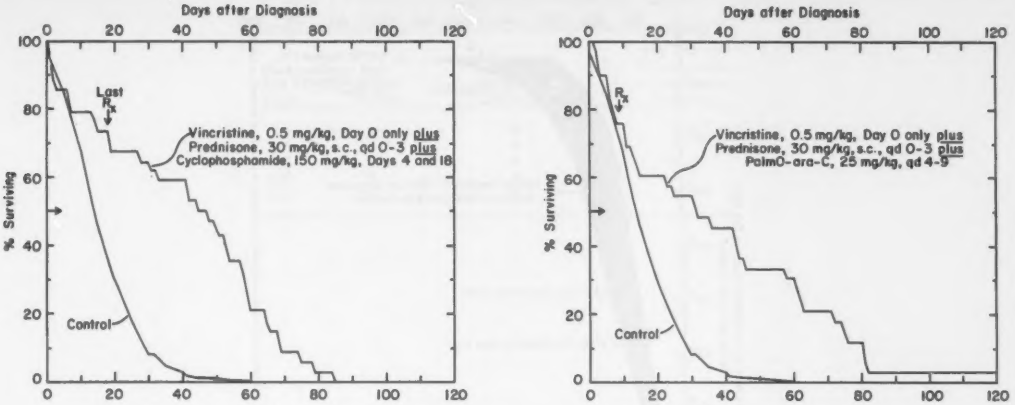
Days after Diagnosis
% Surviving
Last Rx
Vincristine, 0.5 mg/kg, Day 0 only plus Prednisone, 30 mg/kg, s.c., qd 0-3 plus Cyclophosphamide, 150 mg/kg, Days 4 and 18
Control
Days after Diagnosis
% Surviving
Rx
Vincristine, 0.5 mg/kg, Day 0 only plus Prednisone, 30 mg/kg, s.c., qd 0-3 plus PalmO-ara-C, 25 mg/kg, qd 4-9
Control

FIGURE 12.—Survival time plots of therapeutic trial results obtained with vincristine plus prednisone plus cyclophosphamide (trial 306) and vincristine plus prednisone plus palmO-ara-C (trial 310) (*see* table 14).

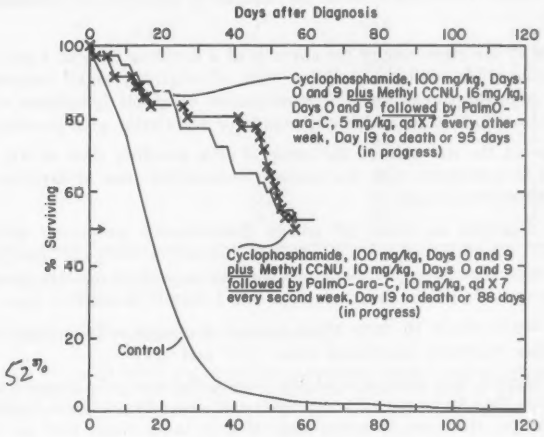
Days after Diagnosis
% Surviving
Cyclophosphamide, 100 mg/kg, Days 0 and 9 plus Methyl CCNU, 16 mg/kg, Days 0 and 9 followed by PalmO-ara-C, 5 mg/kg, qd X7 every other week, Day 19 to death or 95 days (in progress)
Cyclophosphamide, 100 mg/kg, Days 0 and 9 plus Methyl CCNU, 10 mg/kg, Days 0 and 9 followed by PalmO-ara-C, 10 mg/kg, qd X7 every second week, Day 19 to death or 88 days (in progress)
Control
52%

FIGURE 13.—Survival time plots of therapeutic trial results obtained with cyclophosphamide plus methyl-CCNU followed by palmO-ara-C (trials 724 and 727) (not presented in table 14—in progress).

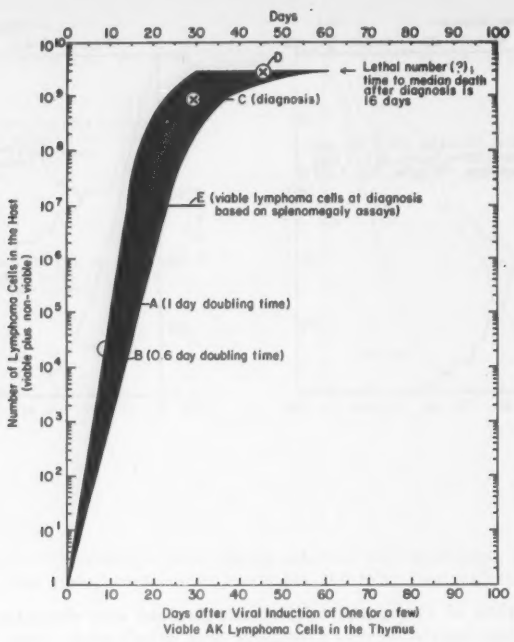
Days
0 10 20 30 40 50 60 70 80 90 100
Lethal number (?); time to median death after diagnosis is 16 days
C (diagnosis)
E (viable lymphoma cells at diagnosis based on splenomegaly assays)
A (1 day doubling time)
B (0.6 day doubling time)
Number of Lymphoma Cells in the Host (viable plus non-viable)
Days after Viral Induction of One (or a few) Viable AK Lymphoma Cells in the Thymus

FIGURE 14.—Approximate rate of growth of spontaneous AK lymphoma from first appearance of viable lymphoma cells in the thymus to diagnosis of widespread disease and to death.

A. The slope of the right side of the curve is at a doubling time of 1 day (up to 10^7) and is compatible with the doubling time of relatively small numbers of first-passage lymphoma cells and the repopulation of viable lymphoma cells after a 4–5-decade reduction by cyclophosphamide or vincristine plus prednisone.

B. The slope of the left side of the curve is at a doubling time of 0.6 day (up to 10^7) and is compatible with the median intermitotic time of dividing lymphoma cells in advanced disease.

C. Clinical diagnosis at about 10^9 widely disseminated lymphoma cells of which about 1% are viable on passage. This is compatible with the observations that the average time lapse between first appearance of viable lymphoma cells in the thymus and clinical diagnosis is about 1 month or slightly less.

D. Median death about 16 days after clinical diagnosis with a lethal number of viable plus nonviable lymphoma cells $> 10^9$ and $< 10^{10}$.

E. Assays suggest that about 1% of the total lymphoma cells present at diagnosis are viable. This value may be somewhat low depending on cell death associated with passage. However, it seems clear that it takes about half as long for 10^9 viable plus nonviable cells to increase to $< 10^{10}$ (16 days) as it does for one or few viable lymphoma cells to proliferate to about 10^9. Cell loss (cell death, lysis, and resorption) must account for most of the slowing of population growth between, say, 10^5 and host death, because the median intermitotic time of the dividing lymphoma cells in advanced disease is 0.6 day and about 97% of the viable cells attempt DNA replication every 24 hours.

Table 1.— Effects of early and delayed thymectomy on the incidence of lymphoma in AKR mice (29).

Age at Thymectomy Days	Age at Thymectomy Months	% of Animals Developing Lymphoma	Avg. Age at Development of Lymphoma (months)
(none; controls)		76	9.1
3	0.1	0	–
35	1.2	8	15.2
150	5	7	11.4
180	6	17	9.8

Table 2.— Typical Splenomegaly Assay Data; Assessment of the Viable Lymphoma Cells in the Thymus of an AKR Mouse with Clinically Diagnosed Lymphoma

Organ Weights of Donor AKR Mouse (mg)		Starting Assay Fraction *	Serial Dilution of Assay Fraction †	Assay Animal No.	Spleen Weight (mg) at One Month	Positive For Viable Lymphoma Cells †		Splenomegaly Units Per Total Thymus
Thymus	Spleen							
470	780	0.01/470	x 1 (none)	1	670	+	3/3	1.8×10^7
				2	540	+		
				3	450	+		
			$\times 10^1$	1	890	+	3/3	
				2	690	+		
				3	635	+		
			$\times 10^2$	1	750	+	3/3	
				2	715	+		
				3	600	+		
			$\times 10^3$	1	610	+	1/3 §	
				2	80	-		
				3	80	-		
			$\times 10^4$	1	90	-	0/3	
				2	85	-		
				3	70	-		

* $\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{mg of donor thymus per recipient in the x 1 dilution}}{\text{thymus wt (mg) of donor mouse}}$

† 0.1 ml of each dilution was inoculated intravenously.

§ A 10^3 dilution of 0.01 mg (0.00001 mg of thymus) contained about 1 viable lymphoma cell (Poisson). Thus, 470 mg/0.00001 mg = 4.7×10^7 viable lymphoma cells; the Fisher Yates Method (38) gives optimal weighting to all serial dilution results and a value of 1.8×10^7 splenomegaly units/thymus.

Table 3.—Splenomegaly Assay Results on the Thymuses of Mice with Clinically Diagnosed AK Lymphoma

Mouse No.	Assay Fraction *	Splenomegaly Dilution Level x 1	x 10^1	x 10^2	x 10^3	x 10^4	x 10^5	Splenomegaly Units/Thymus	95% Confidence Limits Lower	Upper	SU/Thymus (x 100) Avg. Cell No./Thymus
1	0.01/ 470	3/3	3/3	3/3	1/3	0/3		1.79×10^7	4.44×10^6	7.61×10^7	7.0
2	0.01/ 900	3/3	3/3	2/3	2/3	0/3		3.42×10^7	8.51×10^6	1.46×10^8	7.0
3	0.01/ 480	3/3	0/3	2/3	0/3	0/3		4.25×10^5	9.12×10^4	1.26×10^6	0.16
4	0.01/1070	3/3	2/3	0/3	0/3	0/3		9.47×10^5	2.03×10^5	2.81×10^6	0.16
5	0.01/1260	3/3	2/3	0/3	0/3	0/3		1.12×10^6	2.39×10^5	2.81×10^6	0.19
6	0.01/ 730	3/3	2/3	0/3	1/3	0/3		1.26×10^6	3.14×10^5	5.76×10^6	0.31
7	0.01/ 310	3/3	3/3	2/3	0/3	1/3		5.27×10^6	1.43×10^6	2.41×10^7	3.1
8	0.1 / 800	3/3	3/3	3/3	3/3	0/3	0/3	1.36×10^7	3.5×10^6	6.23×10^7	3.1
9	0.1 / 360	3/3	3/3	2/3	0/3	0/3	0/3	3.11×10^5	6.9×10^4	1.23×10^6	0.16
10	0.1 / 800	3/3	3/3	3/3	3/3	1/3	0/3	4.66×10^7	7.54×10^6	1.3×10^8	11.0
11	0.1 / 540	3/3	3/3	2/3	0/3	0/3	0/3	4.67×10^5	1.04×10^5	1.84×10^6	0.16
12	0.1 / 700	3/3	3/3	2/3	2/3	1/3	0/3	6.05×10^6	4.05×10^6	2.44×10^7	1.7
13	0.1 / 700	3/3	3/3	3/3	3/3	0/3	0/3	1.19×10^7	3.07×10^6	5.45×10^7	3.1
14	0.1 / 640	3/3	3/3	0/3	0/3	0/3	0/3	1.11×10^5	2.8×10^4	4.87×10^5	0.034
15	0.1 / 885	3/3	3/3	3/3	1/3	1/3	0/3	7.65×10^6	5.12×10^6	3.09×10^7	1.2
16	0.1 / 630	3/3	3/3	3/3	2/3	1/3	0/3	1.07×10^7	2.76×10^6	4.91×10^7	3.1
17	0.1 /1190	3/3	3/3	3/3	0/3	0/3	0/3	2.08×10^6	5.53×10^5	9.1×10^6	0.32
18	0.1 / 840	3/3	3/3	3/3	3/3	1/3	0/3	4.9×10^7	7.92×10^6	1.36×10^8	1.1
19	0.1 / 830	3/3	3/3	3/3	3/3	0/3	0/3	1.41×10^7	3.64×10^6	6.47×10^7	3.1
20	0.1 / 950	3/3	3/3	3/3	1/3	0/3	0/3	3.59×10^6	8.86×10^5	1.51×10^7	1.7
21	0.1 /1110	3/3	3/3	0/3	0/3	0/3	0/3	1.92×10^5	4.86×10^4	8.45×10^5	0.031
22	0.1 / 600	3/3	3/3	3/3	3/3	2/3	0/3	5.29×10^7	1.16×10^7	2.27×10^8	16.0
23	0.1 / 630	3/3	2/3	3/3	2/3	0/3	0/3	2.38×10^6	5.88×10^5	1.0×10^7	0.7
24	0.1 /1150	3/3	3/3	3/3	3/3	1/3	0/3	6.7×10^7	1.08×10^7	1.86×10^8	11.0
25	0.1 /1235	3/3	3/3	3/3	3/3	1/3	1/3	7.2×10^7	1.16×10^7	2.0×10^8	11.0
26	0.1 / 205	3/3	2/3	2/3	1/3	0/3	0/3	1.77×10^5	3.94×10^4	6.99×10^5	0.16
							mean	1.62×10^7			
							median	5.66×10^6	±0.63 decades		1.2%

* $\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{mg of thymus per recipient bioassay animal at the 1x dilution}}{\text{thymus weight (mg)}}$; lymphoma cells inoculated intravenously.

When serial dilutions were inoculated into groups of 5 instead of 3 young AKR mice, the results obtained were essentially the same.

Table 4.—A Summary of the Results of Splenomegaly Assays for Viable Lymphoma Cells in the Thymus and Spleen of AKR Mice of 3-12 Months of Age*

Age	Fraction of the Random Sample of Animals Having: Clinically Diagnosable Lymphoma	Viable Lymphoma Cells in: Thymus	Spleen	Number Scored as Having Viable Lymphoma Cells In: Thymus But Not Spleen	Spleen But Not Thymus	Both Thymus and Spleen	% of Undiagnosed Animals: With Viable Lymphoma Cells in Thymus	Without Viable Lymphoma Cells in Thymus
3 months (± 7 days)	0/49	5/49†	0/49	5	0	0	10	90
4 months (± 7 days)	0/51	6/51†	0/51	6	0	0	12	88
5 months (± 7 days)	0/49	5/49†	4/49†	5	4	0	10	90
6 months (± 10 days)	0/47	6/47	4/47	4	2	2	13	87
7 months (± 10 days)	2/48	6/48	5/48	1	0	5	9	91
8 months (± 10 days)	2/49	10/49	3/49	7	0	3	18	82
9 months (± 10 days)	2/49	9/49	7/49	3	1	6	14	86
10 months (± 10 days)	5/50	9/50	6/50	3	0	6	8	92
11 months (± 10 days)	8/52	32/52	22/52	11	1	21	46	54
12 months (± 10 days)	2/46	26/46	12/46	15	1	11	52	48
				60	9 (?) §	54		
				49%	7%	44%		

Note: *Although the above animals were picked at random, it should be emphasized that after AKR mice begin to develop clinically diagnosable lymphoma (from which they die in 14-17 days on the average), the remaining animals represent a naturally selected population.

† These borderline assay results require confirmation.

§ These may represent false-positives in the spleen or false-negatives in the thymus; for detailed results see table 5.

Table 5.—An Analysis of the Results of All-or-None Splenomegaly Assays on Thymuses of AKR Mice of 3-12 Months of Age

	Individual Spleen Weights (mg) of Assay Animals Receiving a Total Thymus (Only Assay Results Scored as Positive)									
Age of Donors:	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.	8 mo.	9 mo.	10 mo.	11 mo.	12 mo.
	210	180	205	445	920	610	1070	680	1450	760
	185	155	150	325	825	495	915	570	1450	750
	170	155	150	250	680	325	900	505	1145	450
	145	155	145	210	620	245	895	455	960	440
	145	150	140	180	325	220	575	250	860	400
		140		140	220	220	565	230	760	320
						215	475	225	720	300
						210	155	160	635	250
						145	150	140	620	245
						140			545	245
									540	225
									525	215
									505	185
									430	185
									400	175
									385	175
									385	175
									360	175
									355	175
									335	170
									285	150
									280	150
									270	150
									200	150
									200	145
									200	145
									190	
									190	
									150	
									150	
									140	
median	170	155	150	230	650	217	575	250	392	185
Scored as + for viable cells	5/49 10%	6/51 12%	5/49 10%	6/47 13%	6/48 13%	10/49 20%	9/49 18%	9/50 18%	32/52 60%	26/46 56%
Clinically Diagnosable	0/49	0/51	0/49	0/47	2/48	2/49	2/49	5/50	8/50	2/46

(The assay spleens scored as positive for thymuses of AKR mice of 3-5 months of age are largely borderline when compared to those of animals of 6-12 months.)

In these ip assays a single viable lymphoma cell may lodge and begin proliferation in any of a number of tissues. Progeny must spread to and proliferate in the spleen in order to produce lymphoma-induced splenomegaly.

Assay spleen weights of >135 mg were scored as positive. In controls (651 male and 454 female AKR mice of 6-8 weeks of age) 1.2% had spleen weights >135 mg. The extreme range in 1105 controls was 40-225 mg. Some of the assay spleen weights below the line (< the extreme range of 225 mg) should be considered questionable.

Table 6.—Pulse and continuous labeling indices on all cells in the thymus and spleen of animals bearing advanced spontaneous lymphoma

		Percent Labeled Cells	
		Thymus	Spleen
Pulse (1 hr) TI		6	10
		5	27
		12	20
		10	9
		2	17
		2	12
		14	11
		2	11
		10	19
		2	4
		7	6
	Mean (%)	6.5	13
24-hr TI (q3hr x 8)		7	14
		16	10
		9	13
		22	12
	Mean (%)	14	12
48-hr TI (q3hr x 16)		22	32
		21	30
	Mean (%)	22	31

The above values were obtained on cells from individual animals.

Table 7.—Reduction in Viable Lymphoma Cells Following Varying In Vivo Exposure to Ara-C (A Drug Which Kills Cells When They Are Replicating Their DNA en route to Division)

Treatment	mg/kg/dose	Schedule	In Terms of LD_{10}	Time of Splenomegaly Assay After Last Treatment	Animal No.	Percent Reduction in Viable Lymphoma Cells in the Thymus *
Ara-C	15	q3hr (x 8)	0.63	1 day	1	0
					2	62
					3	70
					4	87.3
					5	96.4
					6	97.3
					7	99.1
					8	99.95
					9	99.96
					10	>99.999
					median	96.9
Ara-C	15	q3hr (x 16)	1.0	1 day	1	99
					2	99.88
					3	99.985
					4	99.986
					5	99.987
					6	99.993
					7	99.998
					8	>99.999
					9	>99.999
					median	99.987
PalmO-ara-C	150 †	Single dose	0.81	2 days	1	97.3
					2	98.1
					3	98.9
					4	99.2
					5	99.85
					6	99.92
					7	99.95
					8	>99.997
					9	>99.998
					10	>99.999
					median	99.89

*Compared to the median historical control for the year in which the splenomegaly assay was carried out.

†A single dose of 150 mg/kg of PalmO-ara-C provides cytotoxic blood levels of ara-C over about 38 hours.

Table 8.— Effects of Cyclophosphamide on Viable Lymphoma Cells in Different Tissues and the Rate of Repopulation of Lymphoma Cells

Treatment	Animal Numbers	Tissue	Splenomegaly Units (Day 0 = Day of Diagnosis) Day 0; 4 Hr. After R_x, If Any	Day 4	Day 5	Day 8	Day 10	Day 12	Day 16
None	1,2,3	Thymus (total)	4,040,000		66,900,000		43,500,000		
		Spleen (total)	3,060,000		7,740,000		1,870,000		
		Lymph nodes (4)	662,000		2,490,000		6,110,000		
		Marrow (1 femur x 20)	13,860,000		3,840,000		3,460,000		
		Liver (total)	1,830,000		1,940,000		2,940,000		
		Blood (per 2 ml)	491,000		1,440,000		95,600		
		Brain (total)	2,580		5,360		58,800		
		Total (>)	23,940,000		84,355,000		58,034,000		
Cyclophosphamide, 150 mg/kg on Day 0 only	4,6,8,10,12	Thymus (total)	260	<9		692		39,600	52,800,000
	5,7,9,11,13	Thymus (total)	1,630	3		54		1,460,000	23,500
	4,6,8,10,12	Spleen (total)	<15	<11		917		830,000	335,000
	5,7,9,11,13	Spleen (total)	<16	<11		<4		39,800	1,120,000
	4,6,8,10,12	Marrow (1 femur x 20)	<60	<40		23,000		230,000	230,000
	5,7,9,11,13	Marrow (1 femur x 20)	<20	<40		1,140		51,000	1,146,000
	4,6,8,10,12	Brain (total)	<9	143		200		126	<100
	5,7,9,11,13	Brain (total)	<12	<20		142		123	158

Treatment	Animal Numbers	Tissue	Organ Weights (mg) at Periods After Cyclophosphamide Day 0	Day 4	Day 5	Day 8	Day 10	Day 12	Day 16
None	Historical Controls	Thymus	470		760		1,130		
		Spleen	800		900		490		
Cyclophosphamide, 150 mg/kg on Day 0 only	4,6,8,10,12	Thymus	680	70		100		115	150
	5,7,9,11,13	Thymus	945	70		35		170	615
	4,6,8,10,12	Spleen	460	80		240		480	390
	5,7,9,11,13	Spleen	470	80		165		230	1,300

It is emphasized that the control assays are on single AKR mice sacrificed on Day 0 and Days 5 and 10 after diagnosis. In the treated groups 2 animals were assayed on Day 0 (4 hours after treatment) and Days 4, 8, 12, and 16. Graphic illustration of the above data is presented in Figure 6.

Table 9.—Some Base-Line Data on Spontaneous AK Lymphoma at Clinical Diagnosis and Death; Median Values and Ranges

Value	No. of Animals	At Diagnosis: Median	At Diagnosis: Range Covering 68% of Values	At Diagnosis: Range Covering 95% of Values
Total WBC Count	1,544	29,500/cu mm	18,700 - 47,500	7,500 - 98,900
Thymus Weights (mg)	120	628	310 - 940	195 - 1,400
Spleen Weights (mg)	120	510	380 - 740	260 - 1,130
No. of Cells per mg Thymus	120	550,000	381,000 - 1,013,000	280,000 - 1,263,000
Splenomegaly Units/Thymus (1969)*	49	2.84×10^6	3.1×10^5 - 1.4×10^7	8.8×10^4 - 6.7×10^7
Splenomegaly Units/Thymus (1970)†	52	5.25×10^5	2.4×10^4 - 3.6×10^6	3×10^2 - 1.6×10^8
Splenomegaly Units/Thymus (1969 and 1970)	101	1.12×10^6	8.8×10^4 - 1.2×10^7	7.3×10^2 - 9.3×10^7
		At Death [16 Days After Diagnosis (Median)]		
Thymus Weights (mg)	1,538	570	240 - 955	60 - 1,530
Spleen Weights (mg)	1,543	395	220 - 660	110 - 1,115

*Diagnosed by palpation plus the requirement of WBC Counts > 25,000.

†Diagnosed by palpation only.

Table 10. Remission Induction in Spontaneous AK Lymphoma With Vincristine + Prednisone and Rate of Repopulation by Viable Cells

Treatment	Animal No.	Splenomegaly Units/Thymus: Day 0 (Day of Diagnosis)	Splenomegaly Units/Thymus: Day 4 (24 Hr. After end of Rx)	Splenomegaly Units/Thymus: Day 14 (11 Days After end of Rx)	Thymus and Spleen Weights (mg) of the Treated Animals: Day 0 Thymus	Day 0 Spleen	Day 4 Thymus	Day 4 Spleen	Day 14 Thymus	Day 14 Spleen
None (Historical Control)	(52 animals)	525,000*	-	-	628 *	510 *				
Vincristine, 0.5 mg/kg on Day 0 + Prednisone, 30 mg/kg on Days 0, 1, 2, and 3	1, 2		130,000	73,500			85	240	425	225
	3, 4		<4	294,000			110	30	170	460
	5, 6		<5	329,000			90	80	190	575
	7, 8		27,000	6,930,000			160	170	180	300
	9,10		<5	4,470,000			90	40	130	395
	11,12		<3	135			80	30	70	510
	13,14		<4	2,220			100	40	290	350
	15,16		5	3,100			110	20	90	60
	17,18		415	275,000			60	35	320	310
	19,20		346	1,680			50	95	110	220
	median	525,000	ca 5	174,250	628	510	90	40	175	330

Note: See Figure 7 for a plot and estimate of the average doubling time of the clonogenic lymphoma cells during early recurrence.

This remission-inducing regimen of Vincristine + Prednisone provided 53% increase in life span in therapeutic trials.

* Median; for distribution of values in 1970, see Table 9.

Table 11.—A Summary of Data on the Reduction of Viable Lymphoma Cells in the Thymus Following Treatment with a Variety of Agents

Treatment	mg/kg/dose	Schedule	In terms of LD_{10}	Time of Splenomegaly Assay after Last Treatment	No. of Animals Studied	Median Percent Reduction in Viable Lymphoma Cells in the Thymus *
Vincristine	1	Single dose	0.37	4 days	10	99.992
	0.5	Single dose	0.19	4 days	10	99.8
	0.25	Single dose	0.25	4 days	10	74
Prednisone	30	qd x 4	<1.0	1 day	10	96.8
Vincristine + Prednisone	0.5 30	Single dose qd x 4	<1.0	1 day	10	99.999
Cyclophosphamide	150	Single dose	0.5	1 day	10	>99.97
L-Sarcolysin	12	Single dose	0.63	1 day	10	>99.997
BCNU	40	Single dose	1.0	1 day	7	>99.987
CCNU	50	Single dose	1.0	1 day	6	99.975
Methyl CCNU	27	Single dose	0.51	1 day	10	>99.997
5-Fluorouracil	200	Single dose	0.8	1 day	7	99.988
Methotrexate	80	Single dose	0.53	3hr-1 day	6	88
Ara-C	30	Single dose	0.01	1 day	10	0
	10	Single dose	0.003	1 day	10	0
	30	qd (x 5)	<1.0	1 day	9	98.7
	30	qd (x 10)	<1.0	1 day	10	90
	15	q3hr (x 8)	0.63	1 day	10	96.9
	15	q3hr (x 16)	1.0	1 day	10	99.987
PalmO-ara-C	150	Single dose	0.81	2 days	10	99.89
	30	qd (x 5)	0.5	2 days	10	92.4
	60	q4d (x 4)	0.82	2 days	10	>99.77
Daunomycin	24 (i.v.)	Single dose	<1.0	1 day	11	99.8

* Compared to the median splenomegaly units/thymus in large numbers of untreated control mice with spontaneous AK leukemia.

Table 12.—Therapeutic Response of Spontaneous AK Leukemia (Lymphoma) to Single Agents

Therapeutic Trial No.	Agents	mg/kg/dose	Schedule (Day 1 = Day of Diagnosis)	% Surv. in Concurrent Toxicity Control (Normal BDF$_1$ Mice)	% ILS: All Animals from Day of Diagnosis	Fraction Dying During Rx	Median Life Span (days): All Animals: After Diagnosis	All Animals: After End of Rx	Just Those Surviving Rx: After End of Rx	% Surviving After End of Rx (All Animals): 60 days	90 days
1968	Controls	–	–	–	–	–	17	–	–	0.4	0.2
1969	Controls	–	–	–	–	–	16	–	–	0	0
1970	Controls	–	–	–	–	–	14	–	–	0.16	0
1971	Controls	–	–	–	–	–	17	–	–	1.9	1.1
1968-71	Controls (total exp.)	–	–	–	–	–	17	–	–	0.7	0.31
517 (71)	Cyclophosphamide	250	day 1	80	74	5/38	29.5	28.5	33	5	3
399 (70)		200	day 1	100	110	8/33	29.5	28.5	37	9	0
406 (70)		150	day 1	100	64	8/34	23	22	23.5	0	0
407 (70)		100	day 1	100	78	4/34	25	24	28.5	0	0
408 (70)		50	day 1	100	67	4/33	23.5	22.5	28	0	0
410 (70)		150	days 1 and 8	100	167	5/31	37.5	29.5	31.5	13	6
622 (71)		150	days 1 and 15	100	100	6/28	34	19	21	4	0
636 (71)		150	days 1 and 22	90	141	6/31	41	19	23	0	0
614 (71)		24	qd 1-15	90	56	3/31	26.5	11.5	14	0	0
616 (71)		16	qd 1-15	100	50	5/31	25.5	10.5	16.5	0	0
625 (71)		11	qd 1-15	100	18	7/31	20	5	7.5	0	0
428 (70)	L-Sarcolysin	9	day 1	100	21	0/30	17	16	16	0	0
627 (71)		6	day 1	100	35	1/29	23	22	22.5	0	0
639 (71)		4	day 1	100	0	1/30	17	16	16	0	0
712 (71)	Nitrogen mustard	0.9	day 1	100	3	3/30	17.5	16.5	18.5	0	0
714 (71)		0.45	day 1	100	0	0/30	17	16	16	0	0
716 (71)		0.22	day 1	100	0	1/30	17	16	16	0	0
373 (70)	MeCCNU	27	day 1	100	57	1/10	22	21	24	0	0
281 (69)		15	days 1, 5, 9, 13	100	31	2/24	21	8	10.5	0	0
431 (70)	BCNU	30	day 1	100	42	2/33	20	19	19	3	3
432 (70)		20	day 1	100	35	0/34	19	18	18	0	0
405 (70)		15	days 1, 5, 9, 13	100	32	10/33	18.5	5.5	7	0	0
461 (70)	CCNU	40	day 1	70	35	3/40	19	18	19	0	0
467 (70)		30	day 1	100	42	0/26	20	19	19	12	8
409 (70)		15	days 1, 5, 9, 13	100	35	12/35	19	6	7	0	0
599 (71)	Ara-C	15	q3hr (x 8); days 1, 5, 9, 13	100	74	10/33	29.5	15.5	20	3	0
333 (70)		10	q3hr (x 8); days 1, 5, 9, 13	100	92	5/35	27	13	16.5	0	0
334 (70)		7.5	q3hr (x 8); days 1, 5, 9, 13	–	114	6/33	30	16	17	6	0
349 (70)		5.0	q3hr (x 8); days 1, 5, 9, 13	–	128	8/34	32	18	20	3	0
366 (70)		2.5	q3hr (x 8); days 1, 5, 9, 13	–	21	16/34	17	3	11.5	3	0
346 (70)		20	q6hr (x 4); days 1, 5, 9, 13	100	96	5/35	27.5	13.5	14	0	0
354 (70)		15	q6hr (x 4); days 1, 5, 9, 13	–	125	4/36	31.5	17.5	21	0	0
357 (70)		10	q6hr (x 4); days 1, 5, 9, 13	–	35	11/30	19	5	12	0	0
367 (70)		5	q6hr (x 4); days 1, 5, 9, 13	–	85	7/34	26	12	14	0	0
344 (70)		40	q12hr (x 2); days 1, 5, 9, 13	100	21	14/32	17	3	15	0	0
350 (70)		30	q12hr (x 2); days 1, 5, 9, 13	–	42	11/34	20	6	8.5	0	0
369 (70)		20	q12hr (x 2); days 1, 5, 9, 13	–	53	10/35	21.5	7.5	10	0	0
370 (70)		10	q12hr (x 2); days 1, 5, 9, 13	–	21	14/35	17	3	13	3	3
324 (70)		40	qd 1-15 days	100	100	2/35	28	13	13	3	0
358 (70)		30	qd 1-15 days	100	92	8/34	27	12	15	0	0
362 (70)		20	qd 1-15 days	–	67	22/68	23.5	8.5	12.5	0	0
364 (70)		10	qd 1-15 days	–	67	10/28	23.5	8.5	13	0	0

393 (70)	PalmO-ara-C	150	day 1	100	89	6/35	26.5	25.5	28	6	0
394 (70)		100	day 1	100	71	4/35	24	23	26	3	0
396 (70)		50	day 1	100	39	2/35	19.5	18.5	19	0	0
397 (70)		25	day 1	100	25	3/35	17.5	16.5	17	0	0
345 (70)		30	qd 1-5 days	-	142	1/34	34	29	29	18	15
378 (70)		30	qd 1-5 days	-	89	5/33	26.5	21.5	24	0	0
304 (70)		25	qd 1-5 days	100	67	9/57	23.5	18.5	23	0	0
353 (70)		20	qd 1-5 days	-	82	4/31	25.5	20.5	21.5	6	0
330 (70)		20	qd 1-15 days	80	92	18/68	27	12	20	1	0
372 (70)		15	qd 1-15 days	100	78	15/42	25	10	12.5	0	0
560 (71)		10	qd 1-15 days	100	60	7/51	27	12	14	0	0
341 (70)		10	qd 1-15 days	100	153	1/34	35.5	20.5	21	3	0
352 (70)		5	qd 1-15 days	100	85	9/33	26	11	15	0	0
355 (70)		1	qd 1-15 days	-	0	21/31	8	during R_x	15	0	0
603 (71)		10	qd 1-30 days	100	62	18/31	27.5	during R_x	11	0	0
509 (70)		100	days 1, 9, 17, 25	80	142	11/34	34	9	22	12	9
510 (70)		67	days 1, 9, 17, 25	100	60	22/35	22.5	during R_x	9	0	0
592 (71)	Amethopterin	0.75	qd 1-15 days	100	29	13/31	22	7	15.5	3	3
602 (71)		0.5	qd 1-15 days	100	24	9/33	21	6	9.5	3	3
610 (71)		0.25	qd 1-15 days	100	10	12/30	19	4	12	3	3
763 (71)		0.25	qd 1-15 days	100	18	13/30	20	5		in progress	
590 (71)		6	days 1, 5, 9, 13	100	47	11/33	25	12	20	3	0
591 (71)		4	days 1, 5, 9, 13	100	15	11/33	19.5	6.5	10	0	0
486 (70)	5-FU	10	qd 1-15 days	100	46	12/31	20.5	5.5	9	0	0
562 (71)		60	days 1, 5, 9, 13	100	53	11/34	26	13	24	3	0
580 (71)		45	days 1, 5, 9, 13	100	29	12/33	22	9	13	0	0
581 (71)		30	days 1, 5, 9, 13	100	35	6/32	23	10	12	3	0
645 (71)	Vincristine	0.15	qd 1-10 days	90	12	5/31	19	9	11.5	6	3
654 (71)		0.10	qd 1-10 days	100	21	4/31	20.5	10.5	12	6	3
661 (71)		0.06	qd 1-10 days	100	29	5/31	22	12	15	3	3
662 (71)		0.04	qd 1-10 days	100	32	6/31	22.5	12.5	16	13	13
693 (71)		0.5	days 1, 8, 15, 22, 29	100	68	18/30	28.5	during R_x	14.5	3	in progress
731 (71)		0.25	days 1, 8, 15, 22, 29	100	38	21/30	23.5	during R_x	in progress		
680 (71)	Prednisone	60	qd 1-4 days	90	18	0/29	20	16	16	0	0
672 (71)		40	qd 1-4 days	100	0	4/31	14.5	10.5	13	3	3
335 (70)	Dexamethasone	10	qd 1-4 days	100	32	3/41	18.5	14.5	15.5	0	0
331 (70)		6.7	qd 1-4 days	100	17	2/30	16.5	12.5	13.5	0	0
326 (70)		4.5	qd 1-4 days	100	28	3/36	18	14	14	0	0
435 (70)		4.5	qd 1-4 days	100	0	4/26	12	8	9	0	0
565 (71)	Daunomycin	4 (IV)	qd 1-3 days	100	41	0/34	24	21	21	3	3
439 (71)		3.5 (IV)	qd 1-6 days	100	44	1/32	24.5	18.5	19	3	3
574 (71)		2.4 (IV)	qd 1-6 days	100	32	1/31	22.5	16.5	17	0	0
516 (71)	Procarbazine	140	qd 1-15 days	90	6	27/69	18	3	8.5	1	0
513 (71)		100	qd 1-15 days	100	0	31/66	17	2	8.5	0	0
512 (71)		67	qd 1-15 days	100	18	18/65	20	5	7	0	0
697 (71)		48	qd 1-15 days	100	0	14/30	17	2	8	3	3
493 (70)	Actinomycin D	0.03	qd 1-15 days	90	28	16/35	18	3	10.5	0	0
717 (71)		0.02	qd 1-15 days	100	0	18/31	14.5	during R_x	10	in progress	
725 (71)		0.13	qd 1-15 days	100	0	14/31	17	2	14	in progress	

The intraperitoneal route of administration was used in all instances except for prednisone and dexamethasone which were administered subcutaneously, and daunomycin which was given intravenously.

Doses 1.5 times the highest listed above were carried out on all of the above agents (except dexamethasone) and proved to be toxic for animals bearing advanced spontaneous leukemia.

Table 13.—Therapeutic Response of Spontaneous AK Leukemia to Combinations of Two Drugs (Various Schedules); Listed in Order of Decreasing Percent Increase of Host Survival Time

Therapeutic Trial No.	Agents	mg/kg/dose	Schedule (Day 1 = Day of Diagnosis)	% Surv. in Concurrent Toxicity Control (Normal BDF$_1$ Mice)	% ILS; All Animals from Day of Diagnosis	Fraction Dying During R_x	Median Life Span (days)			% Surviving After End of R_x (All Animals)	
							All Animals		Just Those Surviving R_x; After End of R_x		
							After Diagnosis	After End of R_x		60 days	90 days
505	PalmO-ara-C MeCCNU	150 24	day 1 day 5	90	196	3/35	41.5	36.5	37	20	6
666	Cyclophosphamide MeCCNU	100 16	days 1 and 10 days 1 and 10	80	185	4/29	48.5	38.5	50	28	21
498	PalmO-ara-C Cyclophosphamide	150 150	day 1 day 3	100	178	0/35	39	36	36	11	6
699	Cyclophosphamide BCNU	75 20	days 1, 8, 15, 22, 29 days 1, 15, 29	80	174	5/28	46.5	17.5	23	7	in progress
503	PalmO-ara-C MeCCNU	150 24	day 1 day 3	100	167	2/35	37.5	34.5	39	22	11
380	PalmO-ara-C Cyclophosphamide	30 150	qd 1-6 days day 9	100	160	3/31	36.5	27.5	29	3	0
539	PalmO-ara-C Cyclophosphamide	150 150	days 1 and 15 days 3 and 17	70	159	10/31	44	27	50	29	6
379	PalmO-ara-C Cyclophosphamide	30 150	qd 1-6 days day 7	90	157	3/31	36	29	31	13	0
134	Cyclophosphamide Ara-C	100 7.5	days 1 and 13 q3hr (x 3), qd 4-10, 16-22	95	155	23/96	43.5	21.5	29	6	1
133	Cyclophosphamide Ara-C	100 7.5	days 1, 13, 25, 37 q3hr (x 3), qd 4-10, 16-22, 28-34, and 40-46	70	144	53/98	41.5	during R_x	24	6	2
556	Ara-C 6-Thioguanine	10 0.67	q3hr (x 8); days 1, 5, 9, 13 q3hr (x 8); days 1, 5, 9, 13	100	142	9/32	34	20	26	3	0
462	Ara-C 6-Thioguanine	15 0.5	q3hr (x 8); days 1, 5, 9, 13 q3hr (x 8); days 1, 5, 9, 13	90	139	9/39	33.5	19.5	24.5	3	0
499	PalmO-ara-C Cyclophosphamide	150 150	day 1 day 5	100	132	3/32	32.5	27.5	31	9	6
374	PalmO-ara-C MeCCNU	15 27	qd 1-15 days day 20	100	121	6/20	31	11	15	5	0
299	PalmO-ara-C MeCCNU	100 27	days 1 and 10 days 4 and 14	70	121	20/54	31	17	31.5	17	11
115	Cyclophosphamide Ara-C	150 7.5	days 1 and 13 q3hr (x 3); qd 4-10 and 16-22	85	115	30/101	33.5	13.5	38	18	7
500	PalmO-ara-C Cyclophosphamide	150 150	day 1 day 7	100	100	7/31	28	24	24	7	0
668	Cyclophosphamide MeCCNU	67 10	days 1 and 10 days 1 and 10	100	94	5/30	33	24	26	7	0

502	PalmO-ara-C BCNU	150 30	day 1 day 1	100	92	6/32	27	22	33	19	13
538	Ara-C 6-Thioguanine	15 0.75	q3hr (x 8); days 1,5,9,13 q3hr (x 8); days 1,5,9,13	100	91	13/35	32.5	18.5	25	3	3
506	PalmO-ara-C MeCCNU	150 24	day 1 day 1	100	89	5/32	26.5	19.5	21	9	0
501	PalmO-ara-C BCNU	150 30	day 1 day 1	100	89	2/32	26.5	23.5	24.5	6	0
472	Ara-C 6-Thioguanine	10 0.5	q3hr (x 8); days 1,5,9,13 q3hr (x 8); days 1,5,9,13	100	89	10/33	26.5	12.5	19	0	0
112	Cyclophosphamide Methotrexate	150 0.75	days 1 and 13 qd 2-12 days	100	88	8/43	32	20	26	2	0
551	Ara-C 6-Thioguanine	10 1	q3hr (x 8); days 1,5,9,13 q3hr (x 8); days 1,5,9,13	100	82	8/33	31	17	21	3	0
463	Vincristine Prednisone	1 30	days 1 and 10 qd 1-4, 11-14	100	81	7/19	28	14	25	11	0
504	PalmO-ara-C BCNU	150 30	day 1 day 7	90	78	4/34	25	18	19.5	3	0
245	Vincristine Prednisone	1 30	days 1 and 11 qd 1-4 and 11-14	100	78	7/20	28.5	14.5	23	0	0
600	Cyclophosphamide MeCCNU	100 16	day 1 day 1	100	74	1/33	29.5	28.5	28.5	9	6
			(Experience with a Single Remission-inducing Course of Vincristine + Prednisone (or Dexamethasone)								
243	Vincristine Prednisone	1 30	day 1 qd 1-4 days	100	40	22/75	22.5	18.5	25	3	0
468	Vincristine Prednisone	0.5 30	day 1 qd 1-4 days	-	67	10/50	23.5	19.5	23	2	0
469	Vincristine Prednisone	0.25 30	day 1 qd 1-4 days	-	53	4/20	21.5	17.5	18	0	0
328	Vincristine Dexamethasone	0.75 4.5	day 1 qd 1-4 days	-	57	4/21	22	18	23	5	0
327	Vincristine Dexamethasone	0.5 4.5	day 1 qd 1-4 days	100	57	6/38	22	18	21	3	0
329	Vincristine Dexamethasone	0.25 4.5	day 1 qd 1-4 days	100	50	1/32	21	17	17	0	0

Table 14.—Therapeutic Response of Spontaneous AK Leukemia to Combinations of Three or More Drugs (Various Schedules); Listed in Order of Decreasing Percent Increase in Host Survival Time

Therapeutic Trial No.	Agents	mg/kg/dose	Schedule (Day 1 = Day of Diagnosis)	% Surv. in Concurrent Toxicity Control (Normal BDF$_1$ Mice	% ILS; All Animals from Day of Diagnosis	Fraction Dying During R_x	Median Survival Time (days)			% Surviving After End of R_x (All Animals)	
							All Animals		Just Those Surviving R_x; After End of R_x	60 days	90 days
							After Diagnosis	After End of R_x			
185	Vincristine	1.0	days 1 and 7	80	348	22/60	55.5	20.5	38	10	4
	Cyclophosphamide	15	days 9 and 19								
	Ara-C	7.5	q8hr (x 3); qd 11-16 and 21-36								
306	Vincristine	0.5	day 1	70	222	11/34	46.5	27.5	39	6	0
	Prednisone	30	qd 1-4 days								
	Cyclophosphamide	150	days 5 and 19								
316	Vincristine	0.5	day 1	80	221	12/33	45	22	51	27	15
	Prednisone	30	qd 1-4 days								
	Cyclophosphamide	150	day 5								
	PalmO-ara-C	25	qd 8-13 days								
255	Vincristine	1.0	day 1	100	215	5/18	50.5	31.5	39	0	0
	Prednisone	30	qd 1-4 days								
	Ara-C	7.5	q8hr (x 4); days 11-16								
	Cyclophosphamide	150	day 19								
508	Vincristine	0.5	day 1	100	170	8/31	30	28	39	23	10
	Dexamethasone	4.5	qd 1-4 days								
	PalmO-ara-C	150	day 7								
	Cyclophosphamide	150	day 11								
507	Vincristine	0.5	day 1	100	143	7/31	34	25	41.5	26	10
	Dexamethasone	4.5	qd 1-4 days								
	PalmO-ara-C	150	day 7								
	Cyclophosphamide	150	day 9								
310	Vincristine	0.5	day 1	100	138	8/33	32.5	22.5	34	21	3
	Prednisone	30	qd 1-4 days								
	PalmO-ara-C	25	qd 5-11 days								
659	PalmO-ara-C	100	day 1	100	128	2/31	40.5	33.5	34	3	in progress
	Cyclophosphamide	100	day 8								
	MeCCNU	16	day 8								
660	PalmO-ara-C	100	day 1	100	134	4/31	38	30	32	3	in progress
	Cyclophosphamide	67	day 8								
	MeCCNU	16	day 8								
303	Vincristine	0.5	day 1	100	121	25/57	31	15	39.5	4	2
	Prednisone	30	qd 1-4 days								
	PalmO-ara-C	25	qd 11-16 days								
156	Vincristine	0.5	days 1, 5 and 9	100	111	27/68	36	16	29	9	2
	Prednisone	13	days 1, 5 and 9								
	Cyclophosphamide	100	day 11								
	Ara-C	7.5	q8hr (x 3); qd 14-20								
321	Vincristine	0.5	day 1	100	103	14/31	28.5	16.5	48	22	13
	Prednisone	30	qd 1-4 days								
	PalmO-ara-C	25	day 5								
	CCNU	25	day 12								
663	PalmO-ara-C	100	day 1	90	94	4/30	33	25	36.5	0	0
	Cyclophosphamide	67	day 8								
	MeCCNU	10	day 8								
680	Vincristine	0.5	day 1	100	88	6/30	32	28	37	17	in progress
	Dexamethasone	4.5	qd 1-4 days								
	Cyclophosphamide	50	day 1								
	PalmO-ara-C	50	day 1								
283	Vincristine	0.5	day 1	100	75	12/30	24.5	14.5	38	7	7
	Prednisone	30	qd 1-4 days								
	PalmO-ara-C	75	day 7								
	MeCCNU	27	day 10								

Table 15.— Random splenomegaly assays on the thymuses of AKR mice which had been diagnosed (advanced lymphoma), treated with cyclophosphamide plus ara-C, and had survived for 60 days or greater after the end of treatment

Day of Sacrifice (days after end of R_x)	Organ Weights (mg) at Sacrifice		Splenomegaly Units/Thymus
	Thymus	Spleen	
61	60	145	0
64	185	110	180,000
71	60	170	0
86	120	240	ca 20
106	120	210	0
129	100	50	3,440
134	630	540	241,000
142	30	240	ca 12
142	40	50	0
155	220	340	0
158	1130	200	2,000
169	60	80	0
			6/12 negative

The rate of repopulation of viable lymphoma cells after cessation of treatment is indicated in figures 6 and 7 (doubling time 0.7-1.0 day).

Table 16.—All-or-None Splenomegaly Assays on Tissues of Mice with Spontaneous Lymphoma Following Treatment

Treatment	Time of Assay After Last Treatment	Animal No.	Assay Results (+ = Assay Spleen >135 mg)										
			Blood	Thymus	Spleen	Marrow	Lymph Nodes	Liver	Brain	Lungs	Kidney	Gonads	Any Tissue
Vincristine, 0.5 mg/kg, day 1 only	1 day	1	+	+	+	+	+	+	-	+			+
		2	+	+	+	+	+	+	+	+		+	+
		3	-	+	-	-		-	-	-		-	+
		4	-	+	-	-	-	-	-	+	-	-	+
		5	+	+	+	+	+	+	+	+	+	+	+
		6	+	+	+	+	+	+	+	+	+	+	+
		7	+	+	+	+	+	+	+	+	+	+	+
		8	+	+	+	+	+	+	-	+	+	-	+
		neg./total	2/8	0/8	2/8	2/8	1/7	2/8	4/8	1/8	1/5	3/7	0/8
Vincristine, 0.5 mg/kg, day 1 only + prednisone, 30 mg/kg, qd 1-4	1 day	1	-	-	+	-	-	-	-	+		-	+
		2	-	-	-	-	-	-	-	-	+	-	+
		3	-	+	+	+	-	+	-	+	+		+
		4	-	-	-	+	-	-	+	+	-	-	+
		5	-	+	+	-	-	+	-	+		-	+
		6	-	+	-	-	-	-	+	+	-	-	+
		7	+	+	+	+	+	+	+	+		+	+
		8	-	+	+	+	+	+	-	+	-	-	+
		neg./total	7/8	3/8	3/8	4/8	6/8	4/8	5/8	1/8	3/5	6/7	0/8
Vincristine, 0.5 mg/kg, day 1 only + prednisone, 0.5 mg/kg, qd 1-4 + cyclophosphamide, 150 mg/kg, day 5	1 day	1	-	-	-	-	+	+	-	+	+	-	+
		2	+	-	+	+		+	+	-	+	-	+
		3	-	-	-	-	-	+	-	+			+
		4	-	-	+	-	-	-	-	+	+	-	+
		5	-	+	+	-	-	-	-	-	-		+
		6	-	+	+	+	+	-	+	+	-	-	+
		7	+	+	+	+	-	-	-	+	+		+
		8	-	-	-	-	-	+	-	+	-	-	+
		neg./total	6/8	4/7	3/8	5/8	5/7	4/8	6/8	2/8	3/7	5/5	0/8
Cyclophosphamide, 100 mg/kg, days 1 and 10 + MeCCNU, 16 mg/kg, days 1 and 10	1 day	1	+	+	-	-		+	+				+
		2	-	+	+	+		+	-				+
		3	+	+	-	-		-	-				+
		4	-	+	-	-		+	-				+
		5	-	+	-	-		+	-				+
		6	-	-	-	-		-	-				-
		7	-	-	-	-		-	-				-
		8	+	+	+	+		+	-				+
		9	-	-	-	-		+	-				+
		10	-	+	+	+		+	+				+
		neg./total	7/10	3/10	7/10	7/10		3/10	8/10				2/10
Cyclophosphamide, 150 mg/kg, days 1 and 15 + ara-C, 7.5 mg/kg, q3hr (x 3), qd 4-10 and qd 16-22. (This regimen has produced toxicity in nontumor-bearing mice in earlier experiments, i.e., 3/20 deaths).	1 day	1		+	+	-		-	-				+
		2		+	+	+		-	-				+
		3		-	-	-		-	-				-
		4		-	-	-		-	-				-
		5		-	+	-		-	-				+
		6		-	-	-		-	-				-
		7		-	-	-		-	-				-
		8		-	+	+		+	+				+
		9		+	-	-		-	-				+
		10		-	+	+		-	-				+
		11		+	+	-		+	+				+
		12		-	-	-		-	-				-
		13		-	-	-		-	-				-
		14		-	-	+		-	-				+
		15		-	-	-		+	-				+
		16		-	-	-		-	-				-
		17		-	-	-		-	-				-
		18		-	-	-		-	+				+
		19		-	-	-		-	-				-
		20		-	-	-		-	+				+
		neg./total		16/20	14/20	16/20		17/20	16/20				9/20

Tissues were suspended by forcing through an 18 gauge needle. The amounts of tissue inoculated i.p. were:
(1) Total thymus into one assay animal.
(2) Total spleen into one assay animal.
(3) Marrow from 2 femurs into a single assay animal.
(4) 1/2 to 1 ml. of blood into a single assay animal.
(5) Approximately 1/4 of total liver into a single assay animal.
(6) Total brain into one assay animal.
(7) 2-4 lymph nodes into one assay animal.
(8) Both lungs, kidneys, testicles or ovaries into one assay animal.

The approximate numbers of viable lymphoma cells in various tissues of untreated animals (as determined by quantitative splenomegaly assay) may be found in Tables 3, 8, and 9.

Table 17.—Prevention or Delay in Appearance of Spontaneous Leukemia (Lymphoma) in AKR Mice

Investigators	Treatment	Deaths from Lymphoma				Comment
		Control		Treated		
		Age	%	Age	%	
McEndy, Boon and Furth (28)	Thymectomy at 1-2 months of age	>17 mo	61-77	>17 mo	8-11	Repeatedly confirmed by other investigators.
Nakakuki et al (29)	Thymectomy at 3 days of age Thymectomy at 35 days of age Thymectomy at 150 days of age Thymectomy at 180 days of age	>17 mo	73-85	>17 mo	0 5-13 6-7 17	Thymectomy as late as 6 months markedly reduced the incidence of lymphoma.
Pollard and Sharon (49, 50)	Cyclophosphamide, 80 mg/kg/week over 8 mo (age 5-13 mo). Germfree AKR mice were employed; this regimen is toxic to conventional AKR mice.	13 mo	88	13 mo	0	Treated animals showed no histologic evidence of lymphoma at 13 months but began to develop lymphoma on cessation of treatment.
Holland (22)	Immunized repeatedly with neuraminidase-treated leukemic AK thymocytes from 2nd to 4th month of age.	15 mo	95	15 mo	65	A promising approach, i.e., following effective chemotherapy.
Schabel et al (unpublished)	PalmO-ara-C, 10 mg/kg/day, qd (x 14) with 2-week intervals of rest, age 6-13 mo.	13 mo	80	13 mo	50	Some toxicity; survivors showed no resistance to challenge with 10^5-10^6 AK leukemia cells.
Schabel et al (unpublished)	PalmO-ara-C, 5 mg/kg/day, qd (x 7) with 1-week intervals of rest, age 6-11 mo of age (expt. in progress)	11 mo	63	11 mo	30	Experiment still in progress; PalmO-ara-C is effective in delaying appearance of clinical lymphoma.

Table 18.—Some Differences Between Murine Leukemia Models and Human Leukemias

	Murine			Human
	Early L1210 Leukemia	Early First-Passage AK Leukemia	Spontaneous AK Leukemia	Acute Leukemia
1. Virus induction	No; transplanted	No; transplanted	yes	? (possible)
2. Average doubling time over the range of about 1 to 10^6 or > leukemic cells; or from induction to diagnosis	0.4-0.5 day	1 day	ca 1 day	?
3. Number of leukemic cells at usual initiation of R_x	1,000,000	1,000,000	1,000,000,000	1,000,000,000,000
4. Number of leukemic cells which are viable (on passage)	ca 50%	?	ca 1%	?
5. Shape of growth curve at usual initiation of R_x	almost exponential	almost exponential	asymptotic	asymptotic
6. Fraction of viable leukemia cells attempting DNA replication within 24 hours	99.999%	ca 99.999%	ca 97%	? Probably closer to spontaneous AK than transplanted murine leukemias
7. Pulse labeling index (all cells)	65%	?	ca 5-10%	ca 5-10%
8. Median intermitotic time (T_C) of dividing cells	0.4-0.5 day	?	0.6 day	ca 2.5 day
9. Median T_S	8 hr	?	8 hr	ca 20 hr
10. Doubling time during repopulation after R_x	0.5 day	?	0.7-1 day	?: ca 4 days at beginning of relapse; probably <4 days earlier
11. Lethal number (viable plus nonviable)	ca 10^9	ca 10^9	slightly $>10^9$	slightly $>10^{12}$
12. Median time from inoculation or diagnosis to death (no treatment)	10^5 IP = 8-9 days	10^6 IV = 20 days (ca 1% viable)	16 days	90 days
13. Reinduction if "cell cure" is achieved	No	Yes	Yes	? (Not as immediate and prevalent a problem as with leukemia in the AKR mouse)

	Host Differences (Approximate)	
	Mouse	Man
1. Weight	20 grams	60 kilograms
2. Body surface area	0.0066 sq. m.	1.6 sq. m.
3. Usual relative tolerated dosage relationships for antileukemic drugs:		
(a) mg/kg	12	1
(b) mg/m^2	1	1
4. Usual maximum serum levels achieved:		
(a) same dose on a mg/kg basis	1	1
(b) same dose on a mg/m^2 basis	ca 12	1
5. Relative serum half life of chemotherapeutic agents	Often of the order of 20-30 minutes	Generally significantly longer

References: 3, 4, 7, 10, 13, 16, 17, 18, 21, 24, 25, 26, 27, 43, 53, and data presented in this report.

Metabolism of Daunomycin (NSC-82151) In Vitro and the Chemotherapeutic Activity of Isolated Metabolites In Vivo [1,2,3]

Mary Ann Asbell, Elaine Schwartzbach, Isidore Wodinsky, and David W. Yesair [4,5]

SUMMARY

Daunomycin (D_1) was quantitatively converted to a metabolite, D_2 (> 98% conversion), by an aerobic, cell-free system containing nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and the 100,000 × *g* supernatant fluid of rat kidneys. Under lowered oxygen concentrations, cell homogenates plus NADPH converted D_1 quantitatively to an unknown metabolite, D_x, with D_2 as a possible intermediate. The chromatographic properties of D_x differed from those of D_2 and the aglycones of D_1 and D_2 (D_4 and D_3 respectively). Sufficient quantities of D_2 and D_x were prepared using NADPH-supplemented cell-free preparations from the kidneys of rats to assay their chemotherapeutic activity in vivo. The metabolite D_2 was as effective as daunomycin (D_1) against P388 leukemic cells growing in mice, but only one sixth as active against L1210 leukemic cells growing in suspension culture. The metabolite D_x was much less active than either D_2 or D_1 when assayed against L1210 cells growing in culture and was inactive against P388 cells growing in mice.
[Cancer Chemother Rep 56:315–320, 1972]

Daunomycin is effective against animal and human neoplasms, especially lymphocytic leukemia (1–3). Unfortunately, therapeutic doses of daunomycin produce toxic effects in several organs of man and animals, including bone marrow, gastrointestinal tract, and heart (4–6). In vivo, daunomycin is converted to a metabolite (D_2) which is extractable from the tissues and urine of several animal species (7) and man (8) and from murine neoplasms (7). Di Marco et al (9) reported that daunomycin was converted to a metabolite by an extract of rat liver or kidney. More recently, daunomycin has been reported to be metabolized in vitro to the metabolite D_2 as well as to another metabolite, D_3 (10–12).

The purposes of this study were to investigate the metabolism of daunomycin by cellular components of rat kidneys, to produce quantities of metabolites in vitro, and to determine the chemotherapeutic activity of the enzymatically produced metabolites.

METHODS

Male Sprague-Dawley rats were killed by cervical dislocation. Kidneys were selected for study since it had been reported (10) that the

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[2] Supported by contract PH43-65-61 from Chemotherapy, National Cancer Institute (NCI), National Institutes of Health.

[3] Daunomycin was supplied by Drug Research and Development, Chemotherapy, NCI.

[4] Life Sciences Division, Arthur D. Little, Inc., Cambridge, Mass.

[5] The authors wish to acknowledge the valuable assistance and advice of Drs. P. S. Thayer and C. J. Kensler.

100,000 × *g* supernatant fluid of kidneys had high activity for the conversion of D_1 to D_2. Kidneys were excised and homogenized with a glass Teflon homogenizer in a solution which contained 0.25 M sucrose, 10 millimols $MgCl_2$, and 10 millimols tris-HCl, pH 7.6. This solution was designated sucrose-tris-Mg^{++}.

The ratio of grams of tissue to milliliters of sucrose-tris-Mg^{++} was approximately 1:10. This suspension was centrifuged at 1000 × *g* for 10 minutes to remove whole cells and large cellular fragments. The supernatant fluid (crude cellular homogenate) was subsequently spun at 9000 × *g* for 30 minutes to sediment the mitochondrial fraction. The resulting supernatant fluid was then spun at 100,00 × *g* for 70 minutes to remove the microsomal fraction. The final supernatant fraction was called the soluble cell fraction and was used immediately or after dialysis against 1500 volumes of sucrose-tris-Mg^{++}. Mitochondrial and microsomal pellets were washed in the sucrose-tris-Mg^{++} solution and finally were resuspended in the same solution. Protein concentrations were determined by the method of Gornall et al (13). Aerobic reactions were conducted in open 25-ml flasks. Anaerobic reactions were defined as those conducted in covered 25-ml Erlemeyer flasks through which nitrogen was being continually bubbled. In most experiments, the reaction system contained 3 ml of daunomycin (80 μg/ml), 5 ml of a protein fraction in sucrose-tris-Mg^{++} (3–4 mg protein/ml), and 0.5 ml of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (10 mg/ml), and was incubated for 2 hours at 37 C. Additional details of experiments may be found in the legends to the figures.

The procedures for chromatographically separating daunomycin and its metabolites are as follows: ascending thin-layer chromatography was carried out on 250μ silica gel G or H plates (Brinkmann) in solvent system I, $CHCl_3$: MeOH:HOAc:H_2O (100:50:14:6; v/v/v/v), or II, $CHCl_3$:EtOH:HOAc:H_2O (179:4:5:12; v/v/v/v).

The silica gel plates were activated at 120 C for 1 hour and allowed to cool in a Brinkmann spotting chamber under a stream of nitrogen. After the samples were applied under nitrogen, the plates were equilibrated for 45 minutes in a Brinkmann solvent chamber before being brought into contact with the liquid phase of the solvent system. Solvents were subsequently allowed to ascend 15 cm. After chromatography, the fluorescent areas were detected with an ultraviolet lamp and then were scraped from the plate into conical test tubes. The fluorescent material was eluted from silica gel with a small volume (approximately 1.0 ml) of EtOH. The concentrations of daunomycin equivalents were determined using a Hitachi Perkin-Elmer MPF 2A Fluorescence Spectrophotometer (excitation wavelength of 490 nm and emission spectrum recorded between 520 and 620 nm) and a daunomycin standard curve.

The metabolite (D_2) was prepared by incubating daunomycin (D_1) aerobically with the soluble protein fraction from rat kidney and NADPH. The reaction mixture contained 4000 mg of soluble protein, 20 mg of daunomycin, and 14 mg of NADPH, in a total volume of 150 ml of sucrose-tris-Mg^{++}. The reaction was allowed to proceed for 2 hours at 37 C with shaking. The production of D_r was similarly accomplished except that the crude cellular homogenate replaced the soluble cell fraction and the reaction was conducted under nitrogen rather than aerobically.

In each case the reaction mixtures were extracted twice with an equal volume of chloroform-methanol (2:1; v/v). The organic phases were pooled and evaporated to dryness in vacuo. The residues were dissolved in absolute ethanol and centrifuged to remove insoluble contaminating material. The supernatant fluids were decanted and evaporated, and the residues were treated with absolute ethanol a second time. This procedure was repeated twice more. Aliquots of the ethanolic solutions of metabolites D_2 and D_r were chromatographed on thin layers of silica gel G with solvent systems I and II, and the fluorescence areas of D_1 (starting material) and D_2 and D_r (metabolites) were quantitated spectrofluorometrically as previously described. The purity of metabolites D_2 and D_r was greater than 98%. The ethanolic D_2 was taken to dryness and solubilized in sterile, physiologic saline. The ethanolic D_r was diluted directly with sterile saline. For cytotoxicity testing the daunomycin used was the clinical dose formulation dissolved in ster-

ile physiologic saline. Controls containing ethanol equivalent to that used in the D_2 and D_x solutions were included in the tests.

L1210 leukemic cells were grown in 200 ml of Fisher's medium (14), supplemented with 10% horse serum, 4×10^4 units of penicillin, and 40 mg of streptomycin (NSC-14083). These cultures, 5×10^4 cells/ml, were incubated with varying concentrations of daunomycin and its metabolites. After exposure to drug for 48 hours, aliquots from each drug treatment were taken for cell counts (15).

P388 leukemic cells (10^6 cells/inoculum) were injected intraperitoneally (ip) in female BDF_1 hybrid mice. Mice bearing tumors were inoculated ip with 1 mg/kg of D_1, D_2, or D_x daily for 10 days. Median survival times were determined for drug-treated animals bearing tumors and compared to those for control animals bearing tumors.

RESULTS

As seen in table 1, daunomycin (D_1) was metabolized under aerobic conditions by a soluble cell fraction from kidney; the metabolite is designated D_2 since its R_F is similar to that reported by Bachur and Cradock (10). Figure 1 depicts the rate of this aerobic conversion of D_1 to D_2 by a soluble cell fraction. The mitochondrial fraction did not accomplish this conversion, whereas microsomal fractions

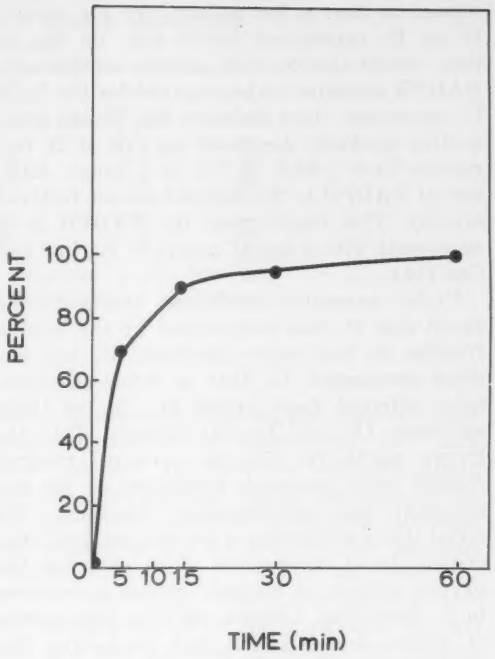

FIGURE 1.—Percent of metabolite (D_2) produced from daunomycin (D_1) by an aerobic soluble cell fraction of rat kidney. The reaction system contained 3 ml of daunomycin (80 μg/ml), 5 ml of soluble protein fraction suspended in sucrose-tris-Mg^{++} at a protein concentration of 3 mg/ml, and 0.5 ml of NADPH (10 mg/ml), and was incubated at 37 C.

TABLE 1.—Aerobic and anaerobic metabolism of daunomycin (D_1) by cell fractions*

	Percent of daunomycin and its metabolites after 2-hour incubation			
	Crude homogenate	Mitochondrial fraction	Microsomal fraction	Soluble fraction
		Aerobic incubation		
Daunomycin D_1	< 1	*ca* 100	85–90	1–2
Metabolite D_2	*ca* 100	< 1	10–15	> 98
Unknown D_x	< 1	< 1	< 1	< 1
		Anaerobic incubation		
Daunomycin D_1	< 1	1–2	5–10	< 1
Metabolite D_2	< 1	< 1	< 1	40
Unknown D_x	> 98	> 98	90–95	60

*The basic reaction systems contained 5 ml of the appropriate cell fraction in sucrose-tris-Mg^{++} at a protein concentration of 3 mg/ml, 3 ml of daunomycin 80 μg/ml), and 0.5 ml of NADPH (10 mg/ml), and were incubated at 37 C for 2 hours. Procedures for anaerobic and aerobic incubations are described in the Methods section.

seemed to have a low activity for the aerobic D_1 to D_2 conversion which may be due to their contamination with soluble constituents. NADPH appeared to be required for the D_1 to D_2 conversion since dialyzing the soluble preparation markedly decreased the rate of D_2 formation from >98% to 5% in 2 hours. Addition of NADPH to the dialyzed sample restored activity. This requirement for NADPH is in agreement with a recent report by Bachur and Gee (12).

Under anaerobic conditions (table 1) we found that D_1 was metabolized by the soluble fraction to two major products, D_2 and another component, D_x, that is chromatographically different from either D_1, D_2, or their aglycones (D_4 and D_3 respectively). This unknown metabolite was the principal product formed under anaerobic conditions by the microsomal and mitochondrial fractions. We noted that maintaining a low oxygen level was critical to D_x production since allowing the oxygen tension to increase caused a decrease in D_x formation. Using a cell-free homogenate of kidney and NADPH and conducting the studies under anaerobic conditions, we found that D_1 appeared to be metabolized first to D_2, which, in turn, was metabolized to the unknown metabolite, D_x (fig 2). The anaerobic metabolism of D_2 (D_2 that was produced separately under aerobic conditions by a soluble cell fraction and isolated) to D_x by all fractions was equivalent to that using D_1 (*see* table 1 for D_1 metabolism). D_x appeared to be an aglycone since it chromatographed at the solvent front in solvent system I and at an R_F of 0.30 in solvent system II. The aglycones of D_1 and D_2 show similar chromatographic behavior, having R_F values in solvent system II of 0.45 and 0.25 respectively. Mixtures of these three aglycones have been separated chromatographically in solvent system II, which indicates that D_x is different from the aglycones of D_1 and D_2.

Our cell-free studies indicated that the extent of conversion of D_1 to D_2 and D_x was such that sufficient quantities of these two components were produced for testing of their chemotherapeutic activity. The relative cytotoxicity of D_1 and D_2 using a 48-hour growth inhibition assay with L1210 in vitro is seen

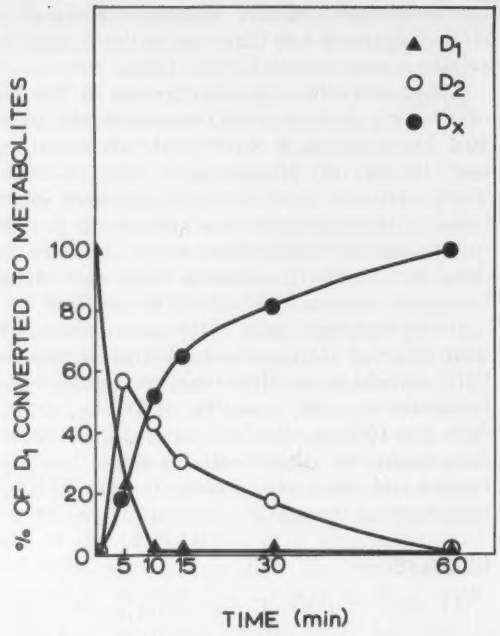

FIGURE 2.—Anaerobic conversion of daunomycin (D_1) to the metabolite (D_x) with D_2 as a probable intermediate. The reaction mixture contained 5 ml of a crude cellular homogenate of rat kidney suspended in sucrose-tris-Mg^{++} at a protein concentration of 3 mg/ml, 3 ml of daunomycin (80 μg/ml), and 0.5 ml of NADPH (10 mg/ml), and was incubated anaerobically (as described in the Methods section) at 37 C. This figure shows the percentages of daunomycin (D_1) and metabolites (D_2 and D_x) in samples withdrawn at the indicated times and quantitated.

in table 2. The daunomycin was the clinical dose form and the 50% inhibition dose was 0.013 μg/ml. The 50% inhibition dose of D_2 was 0.081 μg/ml, or about six times less effective than D_1 against L1210. D_x was much

TABLE 2.—L1210 cytotoxicity assay of daunomycin and metabolites* (48-hour growth inhibition)

Compound	ID50 (μg/ml)
Daunomycin D_1	0.013
Metabolite D_2	0.081
Metabolite D_x	0.933

*The metabolites D_2 and D_x were prepared enzymatically. The clinical dose form of daunomycin was used.

TABLE 3.—Comparative effectiveness of D_1 and D_2 against P388 lymphocytic leukemia*

Compound	Dose (mg/kg)	Change in body weight on Day 5	MST† (days)	% ILS‡
Daunomycin D_1	0	—	11.0	—
	1.00	−1.5	18.0	63
	1.25	−2.2	16.0	45
Metabolite D_2	0	—	10.0	—
	1.00	−3.6	15.5	55

*Female BDF_1 mice that had been inoculated ip with P388 cells were injected daily with 1 mg/kg of daunomycin or metabolite D_2 for 10 days.

†MST = median survival time.

‡ILS = increase in lifespan.

less active than either D_1 or D_2 in this assay system in vitro.

The results with D_2 were similar to those found in studies with D_2 isolated from the urine of rats and dogs (6). The slightly decreased effectiveness of D_2 in those studies was associated with the decreased uptake of D_2 compared to D_1 at equal extracellular concentrations of both. Further, when L1210 cells were grown in suspension culture with daunomycin (0.05 μg/ml) or its metabolite D_2 (0.14 μg/ml) for 4 hours they contained equivalent intracellular concentrations of the compounds (0.2 $\mu g/10^7$ cells). This equivalent intracellular concentration of D_1 or D_2 produced equivalent cytotoxicity, producing a 90%–95% decrease in the cloning efficiency of treated cells compared to untreated L1210 cells.[6]

The chemotherapeutic activities of D_1, D_2, and D_r have also been evaluated against P388 cells growing in vivo. P388 tumor-bearing BDF_1 female mice were treated once a day for 10 days with 1 mg/kg of D_1 or D_2. The results seen in table 3 demonstrate that the D_2 metabolite was equivalent in antileukemic activity to daunomycin (D_1) against P388 leukemia. D_r was inactive against P388 cells in vivo.

DISCUSSION

In tissues of rats, mice, and dogs that have been treated with daunomycin, only a single major metabolite of daunomycin has been found, namely, D_2. However, Bachur's group (10–12) reported that tissue homogenates incubated under anaerobic conditions produced the metabolite D_2, as well as the aglycone metabolites, D_3 and D_4. Under the conditions of our experiments, tissue homogenates converted daunomycin to the metabolite D_2 and also to a new aglycone metabolite, D_r. Mhatre et al (16) reported that an aglycone-like compound was found in tissues of hamsters that had been injected with daunomycin. Others presented evidence that hamsters were more susceptible to daunomycin-induced cardiac malfunction than were a number of other rodent species such as rats (17). Mhatre et al (16) also found that the aglycone of daunomycin (D_4) produced cardiac arrest in isolated dog hearts. We have confirmed the observations of Mhatre et al (16) that an aglycone compound of daunomycin is present in hamster tissues. Further, we have found that this aglycone resembles our D_r (18).

Although D_r was not as chemotherapeutically active in vitro as D_1 and its metabolite D_2, this compound may have considerable chemotherapeutic activity or toxicologic activity or both if produced in situ. We have shown that D_r is present in tissues of rats that have been given daunomycin under hypobaric conditions (18), whereas it would not be present under normal atmospheric conditions. Given this information, it would be interesting to determine whether D_r is produced in L1210 cells exposed to hypobaric conditions. If this con-

[6] Yesair, D. W., Schwartzbach, E., Thayer, P. S., et al. Comparative chemotherapy of daunomycin, its metabolite, and adriamycin. In preparation.

version occurs, then the chemotherapeutic activity of D_r produced in situ could be examined. This possibility of producing D_r in situ may explain the somewhat enhanced chemotherapeutic activity of daunomycin which we have noticed in L1210 cells that are bioassayed by the soft agar cloning technique in which cells remain in soft gel for about 10 days.

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Development of Resistance to Adriamycin (NSC-123127) in Ehrlich Ascites Tumor In Vivo [1,2,3]

Keld Danø [4,5]

SUMMARY

Resistance to adriamycin was developed in an originally adriamycin-sensitive wild strain of Ehrlich ascites tumor (EHR 2) in vivo by treatment with the drug during 25 weekly passages of the tumor. The growth of the resistant line was slower than that of the wild tumor. The resistance was stable for more than 25 weeks both when adriamycin treatment was continued and when treatment was discontinued. The adriamycin-resistant tumor showed cross resistance to daunomycin, just as a daunomycin-resistant subline showed cross resistance to adriamycin. In the adriamycin-resistant tumor, cross resistance to vincristine was also found. There was no change in the sensitivity of the tumor to methotrexate, and it remained nearly insensitive to 6-mercaptopurine, while collateral sensitivity to BCNU and cytosine arabinoside was observed. The data obtained are very similar to data obtained with a daunomycin-resistant line of the same tumor. When adriamycin was given intraperitoneally, it was significantly less toxic than daunomycin, while there was no significant difference in growth-inhibiting effect on the wild Ehrlich ascites tumor.

[Cancer Chemother Rep 56:321–326, 1972]

Adriamycin is an antibiotic isolated from a mutant of *Streptomyces peucetius*. It is an anthracycline and is closely related to daunomycin. These two antibiotics differ in chemical constitution only by substitution of a hydroxyl group (in adriamycin) for a hydrogen atom (in daunomycin) (1,2). Adriamycin has been reported to be more effective than daunomycin in the therapy for several animal tumors, including Ehrlich ascites tumor (1,3). Clinically, adriamycin has been shown to have an effect against acute leukemia and various solid tumors (4).

Previously, we developed resistance to daunomycin in an Ehrlich ascites tumor by prolonged treatment with the drug (5). The resistance was stable as long as daunomycin treatment was continued, but it was gradually reversible when the treatment was discontinued, possibly because of an overgrowth of faster growing sensitive cells present in a mainly resistant tumor cell population (6). Studies on the inhibition of nucleic acid syn-

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[2] Supported by the Danish Medical Research Council.

[3] Adriamycin was supplied by Farmitalia, Milan, Italy. All other drugs used in this study were supplied by Drug Research and Development (formerly Cancer Chemotherapy National Service Center), Chemotherapy, National Cancer Institute. Chemical nomenclature and NSC numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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[5] The author is indebted to Severin Olesen Larsen for assistance with the computer analysis and statistical evaluation. The excellent technical assistance of Miss Inge Strarup, Miss Annette Jacobsen, and Mr. Jørgen Dusing is gratefully acknowledged.

thesis in vitro showed that the resistance to daunomcyin was only relative (7).

In the present study, resistance to adriamycin was developed, and the pattern of sensitivity of the adriamycin-resistant tumor to other drugs was compared with that of the wild tumor and with that of the daunomycin-resistant tumor.

MATERIALS AND METHODS

The experimental design was as described in detail previously (5), with slight modifications.

First-generation hybrids of female random-bred Swiss mice and male inbred DBA mice were used throughout the study.

All drugs were administered intraperitoneally (ip) in isotonic solutions; dosage was based on mouse weight. Adriamycin (as adriamycin hydrochloride) was dissolved just before administration, except when the drug was to be used for development of resistance, in which case dissolved adriamycin, stored frozen at −20 C, was used. Details of other drugs were as described previously (5).

For toxicity experiments, mice were given daily doses for 5 days and were observed for a period of 30 days after the first injection. The LD10, with confidence limits, was calculated by a computerized program of probit analysis (8). When the toxicities of adriamycin and daunomycin were compounded, a program which calculated the potency and its variance by probit analysis was used (8).

The wild tumor studied was EHR 2, a hypotetraploid strain of Ehrlich ascites tumor. Except where otherwise stated, tumors were maintained in mice of alternating sex by weekly ip inoculation with 0.2 ml of undiluted ascitic fluid.

Tumor growth was determined as follows: mice were inoculated with 15×10^6 cells; 7 days after inoculation the total volume of ascites tumor cells in each mouse was determined by removing the ascitic fluid, washing the peritoneal cavity, weighing the pooled ascitic fluid and washings, and determining the cell volume percent by centrifugation in a hematocrit centrifuge. When the ascitic fluid was blood-stained, a correction was made based on the measured hemoglobin content. When experiments were performed with tumors in which resistance was being maintained by adriamycin or daunomycin treatment, the tumor was left without treatment during the week prior to the experiment.

Therapy experiments were performed after randomization of the inoculated mice. The drug was given by daily ip injections for 5 days (starting 24 hours after inoculation), and the total volume of ascites tumor cells per mouse was determined 7 days after inoculation. Mean total tumor cell volume in treated groups was calculated as a percent of the mean in a control group, together with the approximate standard error (SE) of this percentage. A "u" test was performed to evaluate the significance of differences in relative inhibition.

A hemacytometer was used for the counting of cells.

RESULTS

Toxicity

In female mice treated ip daily for 5 days, the LD10 for adriamycin was 2.3 mg/kg (2.2–2.4 mg/kg).[6] A log-probit plot of the data for adriamycin and previous data obtained with daunomycin given under identical conditions is shown in figure 1. It appears that adriamycin under these conditions is less toxic than daunomycin. Statistical evaluation showed that the slopes of the toxicity curves did not differ significantly ($P > 0.20$) while the difference in LD50 (calculated as 1.9 mg/kg [1.6–2.1 mg/kg][6] for daunomycin and 2.9 mg/kg [2.7–3.1 mg/kg][6] for adriamycin) was highly significant (log potency 0.1785 with the variance 0.007, $P < 0.001$).

Therapy for Wild Tumor

The response of the wild tumor to adriamycin is shown as one of the curves in figure 2. Adriamycin inhibits abdominal tumor growth nearly completely at doses below LD10. A corresponding curve for treatment of the wild tumor with daunomycin is shown in figure 3; the inhibiting effect of the two drugs is seen

[6] 95% confidence limits.

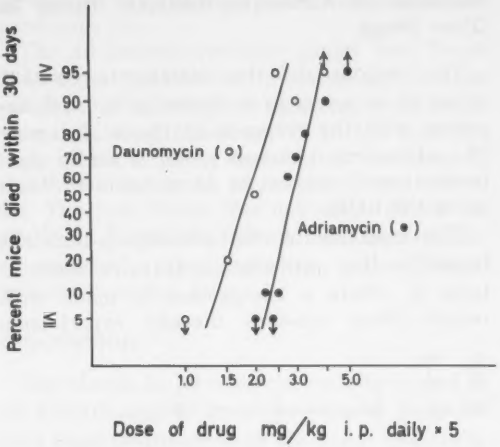

FIGURE 1.—Log-probit plot of toxicity of adriamycin and daunomycin in female mice. Ten mice were tested for each dose, except for daunomycin at 2.5 mg/kg where 20 mice were tested.

to be very similar. For direct comparison of the effect of the two drugs, they were given to randomized groups of mice within the same experiment. As shown in table 1, there were no significant differences in response to the same weight amounts of the two drugs at the two levels tested.

Therapy for Daunomycin-Resistant Tumor

Figure 2 shows also the response of the daunomycin-resistant subline EHR/DM+ to therapy with adriamycin. It appears that this tumor shows almost complete cross resistance to adriamycin in doses approaching the LD10 of the drug.

TABLE 1.—Comparison of growth inhibition of wild Ehrlich ascites tumor with daunomycin and adriamycin

Drug	Dose (mg/kg, ip daily × 5)	Total ascites tumor cell volume in treated mice* (percent of control)	Difference between same dose of the two drugs†
Daunomycin	0.5	34 ± 6	Not significant
Adriamycin	0.5	35 ± 5	(P> 0.20)
Daunomycin	1.0	24 ± 9	Not significant
Adriamycin	1.0	23 ± 5	(P> 0.20)

*Mean value ± SE. There were 12 mice in each test group and in the control group.

† Evaluated by "Student's t" test.

Development of Resistance to Adriamycin

A line of the wild tumor, EHR 2/AM, was maintained in mice by weekly inoculation with 0.2 ml of undiluted ascitic fluid and treatment 5 days a week with adriamycin, starting with a dose of 0.2 mg/kg for eight weekly passages. After this treatment, partial resistance to adriamycin at a dose of 2.1 mg/kg ip daily × 5 was evident. The data from this experiment and the following experiments are given in table 2. The adriamycin dose for development of resistance was then increased to 2.0 mg/kg. After a further ten passages, the tumor was still only partially resistant to adriamycin at a dose of 2.1 mg/kg/day × 5. The cell inoculum used for the weekly transplantations was then reduced by giving 0.2 ml of ascitic fluid diluted 1:8, while the dose of adriamycin was kept unchanged. After seven further passages, the tumor showed complete resistance to treatment with adriamycin at a dose of 2.1 mg/kg/day × 5 (ie, no significant inhibition, $P > 0.20$).

One subline, EHR 2/AM+, was maintained with adriamycin at a dose of 2.1 mg/kg, while

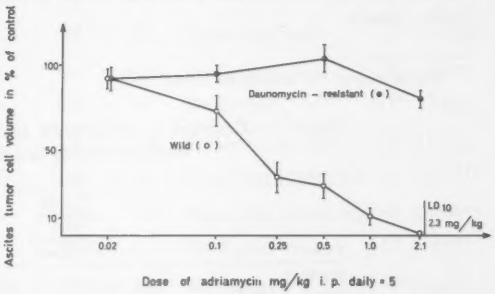

FIGURE 2.—Response to treatment with adriamycin of a wild Ehrlich ascites tumor and of a subline selected for resistance to daunomycin. Each dot represents the mean total ascites tumor cell volume 7 days after inoculation with 15×10^6 cells in 8–12 treated mice (percent of mean total ascites tumor cell volume in 18–19 control mice). Vectors indicate SE of the mean values. Response of the daunomycin-resistant tumor to daunomycin at 1.25 mg/kg ip daily × 5 in the same experiment was inhibition to 98% ± 5% (mean ± SE) of control.

another subline, EHR 2/AM−, was maintained without treatment. Both lines (now in the 26th and 25th passages, respectively, after first demonstration of complete resistance) have retained their resistance.

Growth of Adriamycin-Resistant Tumor

Ip growth of the untreated adriamycin-resistant tumor was determined in an experiment in which ten mice were inoculated with 15×10^6 tumor cells. Seven days later the mean ascites tumor cell number per mouse was found to be $401 \times 10^6 \pm 32 \times 10^6$ cells,[7] with a mean total cell volume of 0.92 ml ± 0.06 ml.[7] Previously it had been found (5) that the wild tumor (under identical conditions) grew to $493 \times 10^6 \pm 29 \times 10^6$ cells,[7] with a total cell volume of 1.39 ml ± 0.08 ml,[7] while a daunomycin-resistant subline grew to $422 \times 10^6 \pm 18 \times 10^6$ cells,[7] with a total cell volume of 1.00 ml ± 0.04 ml.[7] Evaluated by the "Student's t" test this means that the adriamycin-resistant tumor grows to significantly fewer ascites tumor cells ($P < 0.05$), with a smaller total tumor cell volume ($P < 0.001$), than the wild tumor, while neither parameter is significantly different from those found for the daunomycin-resistant subline of the same tumor strain ($P > 0.20$ in both cases).

[7] Mean value ± standard error (SE).

Response of Adriamycin-Resistant Tumor to Other Drugs

The response of the adriamycin-resistant tumor to daunomycin is shown in figure 3, together with the response of the wild tumor. The adriamycin-resistant tumor is nearly completely cross resistant to daunomycin in doses up to the LD10.

The response of the adriamycin-resistant tumor to other antileukemic drugs is shown in table 3, where a comparison is made with results from previous therapy experiments

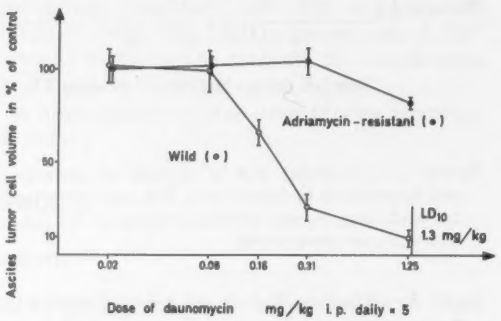

FIGURE 3.—Response to treatment with daunomycin of a wild Ehrlich ascites tumor and of a subline selected for resistance to adriamycin. For experimental conditions and explanation of signs *see* legend for figure 2. Response of the adriamycin-resistant tumor to adriamycin at 2.1 mg/kg ip daily × 5 in the same experiment was inhibition to 88% ± 6% (mean value ± SE) of control.

TABLE 2.—Response to adriamycin treatment of a wild Ehrlich ascites tumor and of sublines during and after development of resistance

Tumor*	Weekly passages with adriamycin treatment	Weekly passages after first demonstration of complete resistance to adriamycin	Total ascites tumor cell volume in mice treated with adriamycin, 2.1 mg/kg ip daily × 5 † (percent of control)
EHR 2	—	—	1.5 ± 0.1
EHR 2/AM	8	—	63 ± 7
"	18	—	67 ± 9
"	25	0	93 ± 9
EHR 2/AM+	51	26	100 ± 11
EHR 2/AM−	—	25	92 ± 9

*EHR 2 is the wild tumor; EHR 2/AM is a subline during development of resistance by treatment with adriamycin (*see* text for doses); EHR 2/AM+ is a subline in which adriamycin treatment was continued after development of complete resistance; EHR 2/AM− is a subline in which adriamycin treatment was discontinued.

†Mean value ± SE. There were 8–12 mice in each test group and 10–18 mice in each control group.

performed with the wild tumor under identical conditions (5).

The adriamycin-resistant tumor was found to be cross resistant to vincristine and to have collateral sensitivity to cytosine arabinoside with both doses tested and to BCNU with the higher dose. With the doses tested there was no significant change in sensitivity to methotrexate. The wild tumor was not inhibited significantly by 6-mercaptopurine, and the adriamycin-resistant tumor showed no collateral sensitivity to this drug.

DISCUSSION

Resistance to adriamycin was developed in an Ehrlich ascites tumor in vivo by intensive long term treatment with the drug. The resistance was stable for more than 25 weeks, both when adriamycin treatment was continued and when it was discontinued. In a previous study, a tumor in which resistance to daunomycin had been developed (and daunomycin treatment continued for 46 weeks), did not lose its resistance after daunomycin treatment had been discontinued for 30 weeks, but after this, the tumor gradually lost resistance (5). Therefore, it is too early to evaluate whether the adriamycin resistance is more stable than the daunomycin resistance. As in the case of daunomycin resistance, ip growth of the adriamycin-resistant tumor was slower than that of the wild tumor.

The tumor line selected for resistance to adriamycin showed cross resistance to daunomycin, just as the line selected for resistance to daunomycin showed cross resistance to adriamycin. The latter finding is in accordance with studies in vitro by Riehm and Biedler, who found that a hamster cell line selected for resistance to daunomycin was cross resistant to adriamycin (9).

TABLE 3.—Growth inhibition with various antileukemic drugs of a line of Ehrlich ascites tumor selected for resistance to adriamycin, compared with response of corresponding wild tumor*

				Therapy				
					Tumor cell volume in treated mice (percent of control)‡			
Drug	Sex of mice	LD10† (mg/kg, ip daily × 5)		Drug dose (mg/kg, ip daily × 5)	Wild Ehrlich ascites tumor	Adriamycin-resistant Ehrlich ascites tumor	Response of adriamycin-resistant tumor compared with wild tumor§	
Adriamycin	F	2.3	(2.2–2.4)	2.1	1.5 ± 0.1	93 ± 9	Resistance	($P < 0.001$)
Daunomycin	F	1.3	(1.1–1.6)	1.25	7.7 ± 1.6	82 ± 4	Cross resistance	($P < 0.001$)
Vincristine	F	0.33	(0.27–0.40)	0.25	67 ± 7	94 ± 10	Cross resistance	($P < 0.05$)
				0.025	101 ± 10	103 ± 9	No change	($P > 0.20$)
Cytosine arabinoside	F	200	(140–280)	200	16 ± 4	7.0 ± 0.9	Collateral sensitivity	($P < 0.05$)
				10	93 ± 7	48 ± 8	Collateral sensitivity	($P < 0.001$)
BCNU	F	14	(12–16)	15	41 ± 5	2.0 ± 0.4	Collateral sensitivity	($P < 0.001$)
				0.50	103 ± 8	103 ± 8	No change	($P > 0.20$)
Methotrexate	M	1.0	(0.7–1.4)	1.0	1.5 ± 0.3	8.0 ± 3.5	No change	($P > 0.05$)
				0.10	93 ± 7	86 ± 13	No change	($P > 0.20$)
6-Mercapto-purine	M	120	(90–160)	100	91 ± 8	74 ± 7	No change	($P > 0.10$)
				50	101 ± 7	96 ± 10	No change	($P > 0.20$)

*Data for toxicity and for treatment of the wild tumor have been published previously (5) except for data relating to adriamycin.

†Numbers in parentheses indicate 95% confidence limits. For all drugs except BCNU, LD10 is based on a 30-day observation period; for BCNU, a 40-day observation period was used.

‡Mean value ± SE. There were 5–16 mice in each test group and 17–20 mice in each control group.

§Significance of differences in response was evaluated with the "u" test.

The pattern of sensitivity of the adriamycin-resistant tumor to other drugs was quite similar to that of the daunomycin-resistant tumor. As with the daunomycin-resistant tumor, cross resistance to vincristine, no change in sensitivity to methotrexate (compared with the wild tumor), no collateral sensitivity to 6-mercaptopurine, and collateral sensitivity to cytosine arabinoside and BCNU were observed. As was discussed previously for the daunomycin-resistant tumor (5), the collateral sensitivity to these two drugs may be directly related to the slower growth of the resistant tumor.

The findings in this study suggest that, at least to some extent, the mechanism of resistance to adriamycin is similar to the mechanism of resistance to daunomycin.

With ip treatment, the subacute toxicity of adriamycin (evaluated by the death of mice) was significantly and considerably less severe than that of daunomycin. It has been reported previously that the acute toxicity in mice receiving intravenous treatment is greater with adriamycin than with daunomycin (1,10). This difference in relative toxicity suggests differences in the pharmacodynamic behavior of the two drugs, eg, in the rate of uptake from the peritoneal cavity, or in the rate or site of drug metabolism.

The inhibiting effects of adriamycin and daunomycin on ip growth of the wild tumor were not found to differ significantly. This is in accordance with reported findings with HeLa cells in vitro, in which the two drugs had the same long term growth-inhibiting effect (11). It has been reported that with ip administration, adriamycin is more active than daunomycin against a strain of Ehrlich ascites tumor when effect on host survival time is measured (1). The present data are not inconsistent with this, but show that, in our case, the main difference in effect of the two drugs was that adriamycin was less toxic than daunomycin by the route and schedule used.

ADDENDUM

The adriamycin-resistant tumor line in which adriamycin treatment was discontinued (EHR 2/AM−) has now been followed in 50 weekly passages without treatment and the line is still resistant to adriamycin.

LIST OF COMPOUNDS

Adriamycin, hydrochloride: NSC-123127

BCNU: NSC-409962; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Cytosine arabinoside: NSC-63878; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunomycin: NSC-82151; daunorubicine, hydrochloride (NSC–83142)

6-Mercaptopurine: NSC-755; purine-6-thiol, monohydrate

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Vincristine: NSC-67574; leurocristine, sulfate (1:1)

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Influence of the Stage of Advancement of Leukemia L1210 in Mice on the Optimal Schedule of Treatment of Cytosine Arabinoside (NSC-63878)[1,2,3]

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SUMMARY

Treatment with cytosine arabinoside given intraperitoneally (ip) every 3 hours for 24 hours on every fourth day (Q3h/24h/4 d) from 1 day after ip implantation of 10^6 or 10^7 leukemia L1210 cells in mice, was not observed to be superior to the same treatment after implantation of 10^5 cells. Similarly, when cytosine arabinoside therapy was delayed until Day 5 after 10^5 tumor cells were implanted, treatment Q3h/24h/4d did not provide a therapeutic advantage over treatment with a single injection every second or fourth day. A single treatment of the advanced disease with cyclophosphamide did not restore the sensitivity of the disease to the Q3h/24h/4d treatment schedule. Whether treatment with cytosine arabinoside was started on Days 1, 3, or 5, the optimal total dose per day for treatment given once every fourth day was 12–20 times greater than the optimal total dose per day for treatment given in eight equal injections every fourth day. Via the oral route, the Q3h/24h/4d treatment regimen was not superior to one treatment every fourth day from Day 1. The data clearly show that when the initial inoculum is high (10^6 or 10^7 cells) or when the disease is advanced, the response to cytosine arabinoside therapy does not display treatment-schedule dependency for the Q3h/24h/4d schedule.

[Cancer Chemother Rep 56:327–334, 1972]

Dr. Burchenal (1,2) aptly described the general status of cancer chemotherapy as follows: "We have a lot of effective drugs. What we still have to learn is their adequate use."

Experimental data have demonstrated that the specificity of action of an antitumor drug expressed in terms of its relative effect on the host and tumor is not a fixed property of the drug. The efficacy of a drug may be influenced by the manner in which the agent is used and by a variety of host and tumor factors such as: (*a*) schedule of treatment (3–7), (*b*) route of administration (4,8–11), and (*c*) the stage of development of the disease (12,13).

Cytosine arabinoside, after its conversion to the nucleoside triphosphate, has been shown to block DNA synthesis by inhibiting DNA polymerase (14,15). Skipper et al (16,17) provided evidence that cytosine arabinoside is

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[2] Supported by contract PH43-68-1283 from Chemotherapy, National Cancer Institute (NCI); National Institutes of Health.

[3] Cytosine arabinoside: cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C. The compounds used in these studies were supplied by Drug Research and Development (DR&D), Chemotherapy, NCI.

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effective against cells only during the S phase of the cell cycle and showed that intraperitoneal (ip) treatment given every 3 hours on Days 2, 6, 10, and 14 was an optimal or near optimal schedule against the early stage of ip implanted leukemia L1210 in mice. These findings were confirmed by many laboratories, including our own (7).

The current experiments were designed to provide information on the influence of the stage of development of L1210 disease on its response to therapy with various treatment schedules of cytosine arabinoside, an S-phase specific agent.

MATERIALS AND METHODS

The materials and methods were essentially the same as those described in previous reports from this laboratory (18,19). The ascitic form of leukemia L1210 used in these studies was carried by weekly transplantation of tumor cells into the peritoneal cavity of DBA/2 mice. The experimental inocula of leukemic cells were implanted either ip (1×10^5–10^7 cells/mouse) or sc (1×10^6 cells/mouse) into BDF_1 (C57BL/6 female × DBA/2 male) male or female hybrid mice which weighed between 17 and 27 g at the beginning of the experiments. One sex only was used in each experiment.

An aqueous solution of cytosine arabinoside was prepared fresh daily in pyrogen-free distilled water (pH adjusted to approximately 4.5 using 1N sodium hydroxide).

The drug was given either subcutaneously (sc), ip, or orally over a wide range of logarithmically spaced dose levels in the constant volume of 0.01 ml/g of body weight. The various treatment schedules used are indicated in the tables and graphs. The relative effectiveness of alternate routes and treatment schedules was based on the results of treatment with the optimal dose. The optimal dose is defined as the dose producing the maximum percentage increase in median survival time (% ILS) over controls.

In experiments D-1361 and D-1961, where the start of treatment was delayed until Day 5 after tumor implantation, six to ten mice were randomly chosen for transplantation of blood or spleen homogenates into normal mice. Consistent death of these recipients from leukemia ensured that the disease was systemic in the donors by Day 5.

The median survival time (MST) of the treated mice is expressed as the percentage increase in lifespan (percent ILS) over the MST of the untreated leukemic mice.

RESULTS

The results summarized in table 1 show that injections of cytosine arabinoside given ip to leukemic mice every 3 hours for 24 hours (Q3h/24h) on every fourth or second day starting 1 day after ip inoculation of 10^5 L1210 cells, were clearly superior schedules. These schedules produced 50% and 37.5% survivors respectively. One treatment every second day elicited 12% survivors, and none of the other schedules produced survivors. The least effective schedules were daily treatment and treatment every 8 days. Treatment given Q3h/24h/8d was superior to one treatment every 8 days but was comparable to one treatment every fourth day and clearly inferior to treatment Q3h/24h/4d.

When cytosine arabinoside was given orally from Day 1 (table 2), Q3h/24h/4d treatment was not superior to treatment given once every fourth day. Both intermittent schedules, however, were superior to daily oral treatment. It should be noted that no survivors resulted from oral treatment with cytosine arabinoside.

When ip treatment was delayed until Day 5 after tumor implantation (table 1), cytosine arabinoside given once every 2 days or Q3h/24h every second or fourth day, produced comparable results (ILS values of 128%, 133%, and 122% respectively).

Figure 1 summarizes the data from experiment D-1961, in which ip treatment with cytosine arabinoside was started on Days 1, 3, or 5 after implantation of L1210 cells. The results confirm the previous observation (table 1) that cytosine arabinoside given in divided doses every 24 hours every fourth day beginning on Day 5 did not provide an advantage over treatment with a single dose every second or fourth day. Of particular interest, however, was the observation that when treatment was

TABLE 1.—Cytosine arabinoside treatment of early and advanced leukemia L1210*

Treatment schedule	Days 1–9			Days 5–13		
	Optimal dose (mg/kg/day)†	% ILS‡	60-Day S/T§	Optimal dose (mg/kg/day)†	% ILS‡	60-Day S/T§
Daily	39/1	89	0/8	23/1, 39/1	89	0/8
Every 2 days	833/1	178	1/8	833/1	128	0/8
Q3h, every 2 days	40/8	222	3/8	67/8	133	0/8
Every 4 days	2320/1	100	0/8	2320/1	89	0/8
Q3h, every 4 days	184/8	>422	4/8	184/8	122	0/8
Every 8 days	2320/1	67	0/8	2320/1	39	0/8
Q3h, every 8 days	184/8	111	0/8	184/8	61	0/8

*Experiment D-1720: 10^5 ascites cells implanted ip; eight BDF_1 male mice (18–23 g) per group.

†Ip treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day. For example, treatment with 5 mg/kg Q3h/24h is expressed as 40/8.

‡Percent increase in median lifespan over controls. Median survival time of untreated controls = 9 days.

§No. of survivors/total treated.

TABLE 2.—Oral cytosine arabinoside treatment of early leukemia L1210*

Treatment schedule (Days 1–9)	Optimal dose (mg/kg/day)†	% ILS‡
Daily	108/1	61
Every 4 days	833/1	133
Q3h, every 4 days	520/8	150

*Experiment D-1342: 10^5 ascites cell implanted ip; eight mice per test group and 16 mice in untreated control groups (female BDF_1 mice, 17–22 g).

†Oral treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day.

‡Percent increase in median lifespan over controls. Median survival time of untreated controls = 9 days.

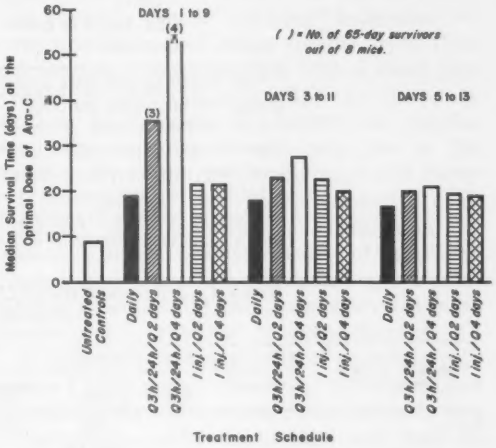

FIGURE 1.—Influence of the day of treatment initiation on the treatment schedule of cytosine arabinoside (ara-C) in mice inoculated ip with leukemia L1210. Eight BDF_1 male mice (19–27 g) per treated group and 16 untreated controls were used. Each bar in the graph represents the optimal response for the indicated schedule of treatment (experiment D-1961).

started on Day 3, the superiority of treatment Q3h/24h/4d over other schedules was substantially reduced.

Summary data from five experiments (table 3) demonstrate that sc treatment with cytosine arabinoside given Q3h/24h/4d was not substantially more effective than treatment every 2 days against advanced sc implanted L1210.

The data presented in table 4 indicate that treatment with cytosine arabinoside given Q3h/24h/4d to mice inoculated ip with 10^5 leukemic cells was clearly superior to the other schedules. However, the therapeutic advantage with this schedule was lost when the leukemic challenge was increased to 10^6 or 10^7 cells.

Figure 2 (experiment D-1961, *see also* fig 1) illustrates the relationship of the total daily optimal dose of cytosine arabinoside to the treatment regimen. Regardless of the day treat-

TABLE 3.—Effectiveness of cytosine arabinoside against advanced leukemia L1210*

Experiment No.	Sc treatment schedule	Optimal dose (mg/kg/day)†	MST‡ (days)	% ILS§
D-1967	Q3h/24h/4d, Days 5–13	50/8,100/8	18	80
	Q2d, Days 5–13	400/1	20	100
	Controls		10	
D-1975	Q3h/24h/4d, Days 5–13	67/8,112/8	20	100
	Q2d, Days 5–13	500/1	21.5	115
	Controls		10	
D-1969	Q3h/24h/4d, Days 5–17	112/8	23	170
	Q2d, Days 5–17	833/1	20.5	141
	Controls		8.5	
D-1889	Q3h/24h/4d, Days 5–21	40/8	22.5	136
	Q2d, Days 5–21	300/1,500/1	21	121
	Controls		9.5	
D-1361	Q3h/24h/4d, Day 5 to death	80/8,160/8	22	144
	Q2d, Day 5 to death	640/1	20.5	128
	Controls		9	

*10^6 L1210 ascites cells implanted sc into eight BDF_1 mice per test group; 16 untreated controls.

†Sc treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day.

‡MST = median survival time.

§Percent increase in median lifespan over controls. Median survival time of untreated controls = 9 days.

TABLE 4.—Influence of size of L1210 inoculum on cytosine arabinoside schedule dependency*

Treatment schedule (Days 1–9)	10^5 Cells			10^6 Cells			10^7 Cells		
	MST†	% ILS‡	60-Day S/T§	MST†	% ILS‡	60-Day S/T§	MST†	% ILS‡	60-Day S/T§
Daily	18	80 (40/1)¶	0/8	18	125 (80/1)¶	0/8	16	129 (40/1)¶	0/8
Every 2 days	23	130 (800/1)¶	0/8	20.5	156 (800/1)¶	0/8	18	157 (800/1)¶	0/8
Every 4 days	21	110 (3200/1)¶	0/8	22	175 (3200/1)¶	0/8	20	186 (3200/1)¶	0/8
Q3h, every 4 days	> 58	> 480 (80/8)¶	4/8	24	200 (80/8)¶	0/8	21	200 (80/8)¶	0/8
Untreated controls	10			8			7		

*Experiment D-2026: L1210 ascites cells implanted ip into eight BDF_1 male mice (19–26 g) per test group; 16 untreated controls.

†MST = median survival time.

‡Percent increase in MST over controls.

§No. of survivors/total treated.

¶ Optimal dose (mg/kg/day). Ip treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day.

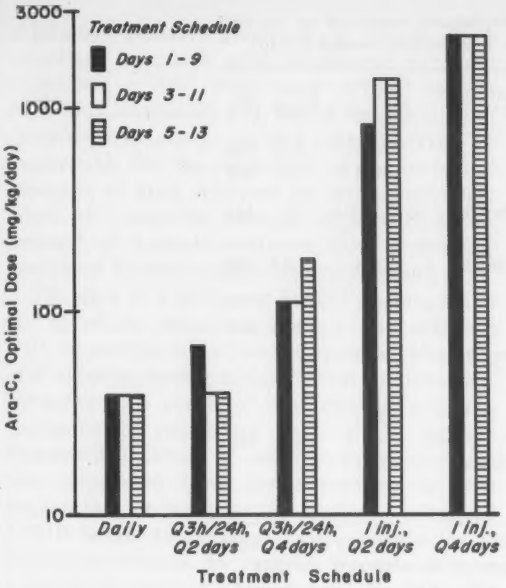

FIGURE 2.—Illustration of the relationship of the total optimal dose on each day of therapy to the schedule of treatment of cytosine arabinoside (ara-C). Treatment with cytosine arabinoside via the various schedules indicated was started either 1, 3, or 5 days after ip inoculation of mice with L1210 cells (experiment D-1961).

ment was started, the total dose on each day of treatment was markedly greater when the treatment was given in a single injection (2300 mg/kg/day given Q4d) as compared with treatment in divided doses (112–184 mg/kg/day given Q3h/24h/4d). It was also observed that the maximally effective dose given once every second day was more than ten times greater than the maximally effective dose on daily administration.

Table 5 summarizes additional data from experiment D-1361 (*see also* table 3, experiment D-1361), in which cytosine arabinoside and cyclophosphamide were given alone and sequentially to mice with advanced L1210. This published study (19) demonstrated the advantage of a priming dose with cyclophosphamide followed by intermittent treatment with cytosine arabinoside over therapy with either agent alone. Of particular interest were the groups of mice that received a priming dose of cyclophosphamide to reduce or attenuate the leukemic population on Day 5 and were then treated from Day 9 with either cytosine arabinoside given Q3h/24h/4d or once every 2 days. In these groups, the enhanced therapeutic responses achieved with sequentially administered cytosine arabinoside were similar (ILS values of 317% and 289%) whether cytosine arabinoside was injected Q3h/24h/4d or once every 2 days after cyclophosphamide.

DISCUSSION

A number of investigators (20–26) have assessed the importance of tumor-cell kinetics to chemotherapy. Basic information obtained from the highly reproducible L1210 leukemia model has been useful in the translation of the implications of these results for clinical trials.

In general, "rapidly growing" leukemias are more responsive to S-phase specific drugs than populations of leukemic cells with a small percentage of dividing cells (21). In the early stage of development of leukemic cell populations, the majority of the cells are in the proliferative stage (21,25). Skipper and Perry (21) pointed out that the proliferative compartment of a leukemic cell population decreases in size with advancement of neoplastic disease, and there is a consistent and unmistakable inverse relationship between advanced disease and "curability" by chemotherapy.

In reference to the growth of leukemic cells in the L1210 model, Shackney (25) presented growth curves which showed that the early rate of growth is nearly exponential and then decreases progressively until an equilibrium population size is reached. With increased doubling times for the leukemic cells during the advanced stage of the disease, response to chemotherapy with S-phase specific agents decreases (21).

Specifically, cytosine arabinoside, an agent whose antileukemic activity is dependent upon the cells being in the S phase of the cell cycle, kills a higher fraction of L1210 cells per given dose or course when the disease is not advanced than when the disease is advanced and the doubling time has increased (21). Skipper et al (16) clearly demonstrated that treatment with cytosine arabinoside given every 3 hours for

TABLE 5.—Influence of initial cyclophosphamide treatment on cytosine arabinoside schedule dependency in mice with advanced L1210*

Agent	Treatment schedule	Optimal dose (mg/kg/day)†	MST‡ (days)	% ILS§
Cyclophosphamide	Day 5 only	300/1	21.5	139
		180/1	19	111
Cytosine arabinoside	Q3h/24h/4d, Day 5 to death	80/8,160/8	22	144
	Q2d, Day 5 to death	640/1	20.5	128
Cyclophosphamide + Cytosine arabinoside	Day 5 only + Q3h/24h/4d, Day 9 to death	180/1 + 160/8	37.5	317
Cyclophosphamide + Cytosine arabinoside	Day 5 only + Q2d, Day 9 to death	180/1 + 320/1	35	289
Untreated controls			9	

*Experiment D-1361: 10^6 L1210 ascites cells implanted sc into eight BDF_1 male mice (19–24 g) per test group; 16 untreated controls.

†Sc treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day.

‡MST = median survival time.

§Percent increase in median lifespan over controls. MST of untreated controls = 9 days.

24 hours with a 3-day interval for host recovery was highly effective in eradicating L1210 cells during the early proliferative stage of the disease. Schabel (22) further elaborated on the "curability" of cytosine arabinoside given every 3 hours on every fourth day against the early stage of ip implanted L1210. However, in mice with a more severe leukemic challenge (10^6 cells injected iv), cytosine arabinoside given Q3h/24h/4d was only moderately more effective than daily therapy with this agent (22). Schabel's observations (22) were confirmed in our studies when mice receiving ip implants with various concentrations of L1210 cells were given cytosine arabinoside via numerous schedules of treatment. Similarly, Shackney (25) stated that the effectiveness of cytosine arabinoside treatment schedules is a function of tumor population size and proposed that a treatment schedule which is effective in early disease may be less effective or ineffective in advanced disease. Our data reported here, which showed that (*a*) the therapeutic advantage of treatment with cytosine arabinoside given Q3h/24h/4d diminished when treatment of mice receiving implants with 10^5 L1210 cells was delayed until Day 3, and (*b*) this schedule provided no advantage over other intermittent regimens when treatment was delayed until Day 5, corroborate the observations of these authors (16,21,22,24,25).

The studies reported here showed that the size of the leukemic inoculum and the day of treatment initiation influenced the efficacy of the Q3h/24h/4d schedule of treatment. The data for oral treatment with cytosine arabinoside suggest that the route of drug administration may be yet another condition for eliciting a superior response with treatment Q3h/24h/4d. It would therefore be of interest to study also the influence of the various routes of drug administration and the sites of tumor inoculation on the response of the host to treatment schedules.

Our data in figure 2 illustrate that the optimal dose of cytosine arabinoside was con-

siderably greater given in a single daily injection compared with treatment in eight injections a day, confirming earlier reports from this laboratory (6). Rapid deamination of cytosine arabinoside (27,28) may, however, be responsible for the magnitude of the increased amounts of drug required for antitumor effect when the interval between injections is increased, eg, a single treatment every second day compared to eight injections every second day.

The data in experiment D-1361 (tables 3 and 5), in which enhanced therapy was achieved with sequential injections of cyclophosphamide and cytosine arabinoside, further indicate the influence the stage of the disease and the leukemic cell population have on the optimal treatment schedule of cytosine arabinoside. It was suggested that the advantage of this sequential chemotherapy against advanced L1210 leukemia resulted from the ability of cyclophosphamide to destroy or attenuate a substantial number of leukemic cells, thereby making the disease more responsive to treatment with cytosine arabinoside (19). Cyclophosphamide did reduce the leukemic cell population, but the tumor kinetics of this population may not have been the same as those of cells in early L1210 disease. As shown in the data, treatment with cytosine arabinoside once every 2 days or Q3h/24h/4d after treatment with cyclophosphamide, gave similar increases in lifespan.

The development of drug-resistant cells among the population of tumor cells being treated could strongly influence the therapeutic efficacy of a given drug. Shackney (25) pointed out that "drug-resistant cell lines might emerge when population size is larger, or when treatment schedules must extend over periods of time longer than 14 days, or both." Rapid development of resistance by L1210 leukemic mice to treatment with cytosine arabinoside has been reported by Wodinsky and Kensler (29) and Dixon and Adamson (30). Data from this laboratory (31) showed that resistance to cytosine arabinoside in mice with advanced L1210 leukemia developed after three or four courses of treatment with this drug given Q3h/24h/4d. In the current studies, large populations of sensitive and resistant leukemic cells could also account for the reduced efficacy of cytosine arabinoside.

Other factors may influence the antitumor effect of this agent when the disease becomes advanced. For example, when proliferating tumor cells become sequestered in "privileged" sites they may not receive adequate exposure to the drug.

Our studies reported here, as well as publications by other investigators, illustrate that the effectiveness of a treatment regimen is closely related to the characteristics of the tumor being treated and have prompted investigations in this laboratory with other schedule-dependent agents.

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Treatment of Chronic Myelogenous Leukemia With 3,3′-Iminodi-1-propanol, Dimethanesulfonate (Ester), *p*-Toluenesulfonate (NSC-140117) Given Orally [1,2]

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SUMMARY

A methanesulfonic acid ester of the aminoglycols, 3,3′-iminodi-1-propanol, dimethanesulfonate (ester), *p*-toluenesulfonate (NSC-140117) has been found to be very effective in inducing and maintaining complete remissions in early cases of chronic myelogenous leukemia. The drug was given in daily doses of 1–2 mg/kg for remission induction and 0.5–1.0 mg/kg for remission maintenance, with dose adjustment according to blood cell counts. It induced complete remissions (normal white blood cell count and complete disappearance of splenomegaly) in all nine patients treated and has been maintaining these remissions (as well as a complete remission which was originally induced by NSC-84641 in an additional patient) for a period of 2⅓+ to 15⅓+ months. The only toxic effect observed occurred in two patients who had mild discomfort in the upper abdomen associated with soft stools. We need further observations, however, regarding the chronic toxic effects of this drug. The myelosuppressive effect of NSC-140117, within the dose range used, is granulocyte specific and appears less long lasting than that of busulfan.

[Cancer Chemother Rep 56:335–338, 1972]

Two newly synthesized sulfonic acid esters of aminoglycols, 3,3′-(methylimino)di-1-propanol, dimethanesulfonate, hydrochloride (NSC-84641) and 3,3′-iminodi-1-propanol, dimethanesulfonate (ester), hydrochloride (NSC-102627), have been found to be remarkably effective in inducing remissions in patients with chronic myelogenous leukemia (CML) (1). Despite their distinct effectiveness, both of these agents are impractical for the treatment of CML because they must be administered intravenously (iv). Recently, oral tablets of NSC-102627 (3,3′-iminodi-1-propanol, dimethanesulfonate (ester), *p*-toluenesulfonate; NSC-140117) (fig 1) have been prepared, and the effect of this oral form of treatment on CML has been assessed.

PATIENTS

As of August 1971, ten patients (eight men and two women), ranging in age from 27 to 55 years, were treated with NSC-140117. Four patients, Nos. 1, 6, 8, and 9, had received no pre-

[1] Received Aug 9, 1971; revised Dec 16, 1971; accepted Jan 26, 1972.

[2] NSC-140117 was supplied by Yoshitomi Pharmaceutical Co., Ltd., Osaka, Japan.

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FIGURE 1.—Chemical structure of NSC-140117.

vious treatment. Patient No. 2 had been treated with NSC-102627 administered iv and patients Nos. 3 and 5 had been treated with NSC-84641 administered iv; complete remission was obtained in each case. Patient No. 7 had received carbazilquinone [4] and had a good but temporary response. Patient No. 4 had been overtreated with busulfan,[5] resulting in severe pancytopenia approximately 9 months before NSC-140117 treatment. In patient No. 10, NSC-140117 was given to maintain a remission which had been induced with NSC-84641 given iv.

DRUG ADMINISTRATION

NSC-140117 was supplied in 10-mg tablets. For remission induction, 1–2 mg/kg of the agent was given daily in two divided doses, and for remission maintenance, 0.5–1.0 mg/kg was given in one dose or two divided doses. Blood cell counts and physical examinations were performed every 1–3 weeks. Liver and kidney functions were checked every 3–4 weeks.

The initial dose for remission induction ranged from 60 to 120 mg/day and the dose for remission maintenance ranged from 30 to 60 mg in most patients. The dose was adjusted or temporarily discontinued according to the white blood cell count (wbc).

[4] Carbazilquinone: NSC-134679; *p*-benzoquinone, 2,5-bis(1-aziridinyl)-3-(2-hydroxy-1-methoxyethyl)-6-methyl-, carbamate (ester).

[5] Busulfan: NSC-750; 1,4-butanediol, dimethanesulfonate.

RESPONSE

In all nine patients receiving remission induction therapy, the wbc counts before treatment ranged from 67,500 to 217,000 cells/mm^3 (median, 130,000). The counts started to show significant reductions after 2–4 weeks (median, 3) and fell to less than 10,000 cells/mm^3 after 7–10 weeks (median, 7) of drug administration. The amount of drug administered during this period ranged from 2080 to 5580 mg (median, 4110).

Palpable splenomegaly which was present in eight of nine patients completely disappeared during the remission induction or the early part of the maintenance therapy. An excellent remission (1) characterized by a complete disappearance of splenomegaly and normal wbc was obtained in all nine patients (table 1).

Hematologic changes during maintenance therapy with NSC-140117 are summarized in table 2. During most of the clinical course, the wbc counts in each patient have been well controlled at around 10,000 cells/mm^3 by adjusting the dosage. The median hemoglobin level has shown a spontaneous rise from 12.3 g/100 ml at the time remission was achieved to a high of 15.7 g/100 ml. Thrombocytopenia was not observed except in patient No. 4 who had been overtreated with busulfan. In this patient the remission was terminated by blastic crisis after 8⅓ months' duration. In the other nine patients excellent remissions have been maintained for 2⅓+ to 15⅓+ months.

TABLE 1.—Treatment of chronic myelogenous leukemia with NSC-140117 given orally: clinical effect

Patient No.	Age, sex	Prior chemotherapy	Wbc × 10^3		Effect of drug on splenomegaly*	Overall effect	Remission duration
			Before therapy	During remission			
1	41, M	—	155.0	9.6	+++	Excellent	12⅔+
2	27, F	NSC-140117, iv	198.5	8.9	+++	Excellent	15+
3	55, F	NSC-84641	130.0	9.8	+++	Excellent	12+
4	39, M	Busulfan	67.5	5.8	+++	Excellent	8⅓
5	27, M	NSC-84641	93.0	6.1	+++	Excellent	13⅔+
6	26, M	—	180.4	5.1	+++	Excellent	13+
7	35, M	Carbazilquinone	112.7	8.6	+++	Excellent	2⅓+
8	38, M	—	217.0	6.9	+++	Excellent	2⅔+
9	47, M	—	100.0	9.8	+++	Excellent	3⅓+
10	44, M	NSC-140117 was used for maintenance of remission induced by NSC-84641 given iv.	—	—	—	—	15⅓+

*+++ = Complete regression of splenomegaly.

TABLE 2.—Treatment of chronic myelogenous leukemia with NSC-140117 given orally: hematologic changes during maintenance therapy

Patient No.	Wbc × 10^3		Hgb level (g/100 ml)		Platelets × 10^3		Remarks
	Highest count	Lowest count	During remission	Highest level	Highest count	Lowest count	
1	14.2	2.6	10.2	15.0	477	128	Epigastric distress, soft stools
2	29.9	4.1	12.3	14.4	1960	436	Slight elevation in SGOT and SGPT levels which became normal during therapy
3	17.3	7.2	12.6	15.7	602	222	Marked elevations in SGOT and SGPT levels which have become normal while resuming therapy
4	58.5	5.8	14.7	17.3	309	32	Temporary thrombocytopenia, epigastric distress; patient had been overtreated with busulfan
5	14.5	3.4	12.6	16.5	1917	453	
6	23.5	2.6	12.0	16.2	537	251	
7	8.6	6.3	10.2	13.3	340	252	
8	6.9	4.5	12.0	15.5	378	140	
9	25.5	9.8	16.3	16.3	156	133	
10	26.5	4.2	14.7	19.8	1122	432	Received venesections for polycythemia

TOXIC EFFECTS

The toxic effects of NSC-140117 observed so far occur infrequently and are mild. In two patients mild discomfort in the upper abdomen accompanied by soft stools was noted, which was easily controllable. Patient No. 3 had marked elevation of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels, which was associated with symptoms of viral hepatitis. NSC-140117 administration was discontinued because of its possible hepatotoxicity. Treatment was resumed when the transaminase levels returned to normal. SGOT and SGPT levels have not been elevated since that time, indicating that NSC-140117 did not contribute to the hepatic damage in this patient. In patient No. 2, slight elevation in SGOT and SGPT levels was noted but the levels gradually returned to normal while she was still receiving treatment. No abnormalities were noted in blood urea nitrogen or serum creatinine levels.

DISCUSSION

Oral administration of NSC-140117 has been shown to be very effective in inducing as well as maintaining complete remissions in early cases of CML. As of August 1971, it had induced excellent remissions in the nine patients who had received remission-induction therapy with this agent, and it has been maintaining these remissions in these nine patients and in patient No. 10, whose remission was previously achieved with NSC-84641 given iv (1).

Within the dose range used, myelosuppression of NSC-140117 appears to be quite granulocyte specific, and in spite of the continuous treatment, hemoglobin levels returned spontaneously to normal ranges. Polycythemia developed in patient No. 7, requiring venesections on two occasions. Thrombocytopenia was not observed except in patient No. 4 who had been overtreated with busulfan.

The only toxic effect attributable so far to the drug is upper abdominal discomfort associated with soft stools, which was observed in two patients. The observation period, however, was not long enough to rule out any chronic toxic effects.

It was noted that the granulocyte-suppressive effect of NSC-140117 is rather transient

and continuous administration is necessary to maintain remissions in most patients. Our impression is that this agent is safer and easier to administer than busulfan for the treatment of CML.

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Failure of Combination Therapy with S-carbamyl-L-cysteine (SCC; NSC-102498) and L-Asparaginase (NSC-109229) for L-Asparaginase-Resistant Mouse Lymphoma [1,2,3]

Morton D. Prager and Louise Rothberg [4]

The antileukemic activity of L-asparaginase apparently stems from L-asparagine depletion of those hosts carrying tumors with inadequate capacity for L-asparagine biosynthesis. In experimental systems there has been a high degree of correlation between sensitivity or resistance to L-asparaginase and the asparagine synthetase (ASase) activity of the malignant neoplasm (1–5). ASase, the only known mammalian enzyme catalyzing asparagine synthesis, does so by the following reaction:

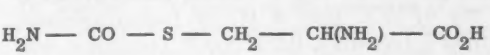
$$\text{L-aspartate} + \text{L-glutamine} + \text{ATP} \xrightarrow{Mg^{+2}} \text{L-asparagine} + \text{L-glutamate} + \text{AMP} + PP_i$$

It seems reasonable to hypothesize, therefore, that an antimetabolite capable of inhibiting ASase might potentiate the L-asparaginase effect, even with tumors normally resistant to the action of the enzyme. A number of L-glutamine antagonists have been examined in combination with L-asparaginase with some therapeutic benefit (6–9). Although L-glutamine antagonists would be expected to have an inhibitory effect on ASase, several that have been studied differ in their potency (10). In the current investigation the amino acid analog S-carbamyl-L-cysteine (SCC) (fig 1) was studied for its ability to inhibit ASase since its effects as an antimetabolite are partially reversed by L-glutamine (11). It was also used alone and in combination with L-asparaginase to treat C3H mice bearing the L-asparaginase-resistant 6C3HED-R lymphoma.

MATERIALS AND METHODS

Asparagine Synthetase Assays

The ASase determination was conducted essentially as previously described (12). In brief, the 1.0 ml of reaction mixture contained 1.5 millimols of ^{14}C-labeled L-aspartate (1.392×10^6 dpm/μmol); 5 millimols of L-glutamine; 10 millimols of $MgCl_2$; 10 millimols of ATP; and 0.1 M tris buffer (pH 8.0). ASase was obtained from 6C3HED-R lymphoma by homogenizing the tumor in the Medium A of Keller and Zamecnik (13) to which dithiothreitol was added to a final concentration of 1 millimol. The supernatant fluid from centrifugation at 41,000 × *g* for 30 minutes (Sorvall RC2-B) was dialyzed against 2 × 250 ml of the Me-



S-carbamyl-L-cysteine

L-Glutamine

FIGURE 1.—Structures of S-carbamyl-L-cysteine (NSC-102498) and L-glutamine.

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[3] S-carbamyl-L-cysteine: cysteine, S-carbamate, monohydrate, L-. Obtained from Dr. William Shive, Department of Chemistry, University of Texas at Austin.

L-Asparaginase was supplied by Merck Sharp and Dohme.

[4] Departments of Surgery and Biochemistry, University of Texas Southwestern Medical School, Dallas.

dium A-dithiothreitol solution, and a 3–5-mg sample of protein (biuret) from this preparation was then used for the ASase assay. After a 30-minute incubation at 37 C, 1.0 ml of 0.8N $HClO_4$ was added to stop the reaction, followed by 0.5 mg of carrier L-asparagine. The solution was brought to pH 6–7 with 2.5N KOH, the precipitate was removed by centrifugation, and the supernatant fluid was chromatographed on a column of Amberlite CG 400 formate. The water wash was collected, and from the specific activity of the eluted L-asparagine the activity of the ASase was calculated. To test the effect of SCC on the ASase reaction, SCC was dissolved in the 0.1 M tris buffer (pH 8.0) at a concentration of 200 millimols, and was added to the ASase assay mixture to give concentrations of either 20 or 5 millimols. One unit of ASase activity is defined as that which produces 1 μmol of L-asparagine/minute.

Treatment of C3H Mice Bearing 6C3HED–R Lymphoma

Twenty-five C3H mice received intraperitoneal (ip) injections of 1×10^6 6C3HED-R (asparaginase-resistant) ascites lymphoma cells. Cell viability was determined by trypan blue exclusion. The mice were divided into five groups for daily treatment with either SCC alone or a combination of SCC and *Escherichia coli* L-asparaginase or neither (control): Group 1 mice received 4 mg of SCC; Group 2, 8 mg of SCC; Group 3, 4 mg of SCC plus 3 International Units (IU) of L-asparaginase; Group 4, 8 mg of SCC plus 3 IU of L-asparaginase; Group 5 (control), 0.9% NaCl. Treatment was by ip injection on 6 successive days beginning on the third day after tumor cell implantation. An L-asparaginase unit is defined as that activity producing 1 μmol of product/minute. The NH_3 liberated in the L-asparaginase reaction was measured by nesslerization (3).

RESULTS AND DISCUSSION

Results presented in table 1 show that SCC was capable of inhibiting the ASase of the 6C3HED-R lymphoma. At SCC to L-glutamine ratios of 4:1 and 1:1, inhibition was 77% and 27%, respectively. To determine the true effect of an inhibitor, it is important to dialyze the cell supernatant fraction used as an ASase source so as to eliminate free L-asparagine which acts as an inhibitor of the enzyme. In this study, the observed ASase activity increased from 2.1×10^{-4} to 2.6×10^{-4} units/mg protein as a result of dialysis. At an apparent SCC to L-glutamine ratio of 4:1 for an undialyzed preparation, the degree of inhibition decreased to 38%, presumably because of the presence of additional free L-glutamine. These results indicate that SCC is capable of significant inhibition of the L-asparagine biosynthetic reaction. However, if the free intracellular L-glutamine concentration is high, the inhibitory effect would be diminished.

TABLE 1.—Inhibition of asparagine synthetase by S-carbamyl-L-cysteine (SCC)

SCC to L-glutamine ratio	Asparagine synthetase	
	Units	Percent inhibition
0:1	2.6×10^{-4}	0
1:1	1.9×10^{-4}	27
4:1	0.6×10^{-4}	77

Despite the inhibitory effect of SCC on the ASase of the lymphoma used in the therapeutic trial, no prolonged survival was achieved in any of the experimental groups (table 2). The dose level of SCC was in the range of that which gave promising results with the RC mammary adenocarcinoma (14) and the P1798 lymphosarcoma (15). In addition to the obvious possibility that the best therapeutic regimen was not found, other reasons for failure to prolong survival might stem from (*a*) Broome's observation (16) that administration of agouti serum L-asparaginase caused an increase in intracellular L-glutamine, (*b*) the observations that L-asparaginase administration may also lead to increased ASase activity (2, 3, 17), and (*c*) the difficulty of achieving a sufficiently high SCC to L-glutamine ratio in vivo.

TABLE 2.—Treatment of C3H mice bearing 6C3HED-R with S-carbamyl-L-cysteine (SCC) alone or in combination with L-asparaginase

Treatment (dose)	No. of mice	Survival (days)	
		Mean	Standard error
None (0.9% NaCl)	5	16.4	0.2
SCC (4 mg)	5	18.2	3.2
SCC (8 mg)	5	15.2	0.5
SCC (4 mg) + L-asparaginase (3 IU)	5	16.2	0.2
SCC (8 mg) + L-asparaginase (3 IU)	5	13.8	1.4

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Clinical, Radiologic, and Histopathologic Studies on Pulmonary Toxicity Induced by Treatment With Bleomycin (NSC-125066) [1,2]

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SUMMARY

This study describes the clinical, roentgenologic, and histopathologic studies of pulmonary toxicity induced by treatment with bleomycin. In 168 patients with evaluable cases who were treated with different dose schedules of bleomycin, the overall incidence of pulmonary toxicity was 39.8%. The mean total dose producing pulmonary damage was about 100–150 mg/m². In 31 patients (46%), pulmonary changes were detected only after systematic auscultation of the lungs (fine crepitant rales at one or both bases). In 36 patients (54%), roentgenograms showed reticular or reticular micronodular lesions located mostly at the lower lung zones and at the costophrenic angle 1–2 weeks after the crepitations were noted. In seven patients postmortem examination revealed hyperplasia and endoalveolar migration of type II pneumocytes and macrophages, fibrinous edema, hyaline membranes, and newly formed reticular and collagen fibers within the alveolar septa. The radiologic and histopathologic findings were consistent with the diagnosis of interstitial pneumonia. These pulmonary reactions represent a delayed dose-dependent bleomycin effect and are probably due to the preferential distribution of the drug in the lung tissue.

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Among the new antitumor antibiotics, bleomycin was recently found to be of particular interest because it appears to cause no significant myelosuppression and has considerable therapeutic activity against malignant lymphomas and epidermoid carcinomas (1–12), particularly those of the head and neck (2,4–7,13) and the esophagus (2,5–7,15).

The drug is a peptide complex which was isolated in Japan by Umezawa (15). It is active in some experimental mouse and rat tumors (16). It inhibits the incorporation of thymidine into the DNA of HeLa cells and *Escherichia coli* cells (17,18), complexes with the DNA molecule, and uncoils its double helix (19,20). Disposition studies have shown that in man, about 40% of the administered dose is excreted in the urine within the first 24 hours after administration (21). After a single intravenous (iv) injection of 15 mg, the blood level falls rapidly during the first 30 minutes, but more slowly thereafter (21). Bleomycin is distributed in high concentrations in tissues of skin, lungs, peritoneum, lymph, and epidermoid tumors (16,21).

In the past few years, Japanese (2,13,22,23),

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[2] Bleomycin was kindly supplied by the Laboratorio Roger-Bellon, Sesto San Giovanni, Italy.

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European (4–7,24), and American (8–11) investigators have reported a number of side effects secondary to bleomycin administration. To a great extent, the toxicity is related to the previously mentioned tissue distribution (thickening of the skin on the fingers, palms, and soles; skin hyperpigmentation; distrophic changes of the nails; alopecia; and pulmonary interstitial fibrosis). Furthermore, pyrexia, especially after the first few injections, stomatitis, gastrointestinal disturbances (nausea, vomiting, and diarrhea), and generalized allergic-like erythema have also been reported.

While the absence of myelosuppression represents the most consistent advantage in the treatment with bleomycin, lung toxicity has been regarded as a limiting factor for an intensive and prolonged course of therapy. Since the incidence of pulmonary damage varies even among reports from Japanese investigators and, to our knowledge, no specific detailed reports on the clinical, radiologic, and histopathologic aspects of this peculiar toxic effect have been published, this paper attempts to describe the incidence, findings, and course of pulmonary toxicity in a group of patients treated with different dose schedules of bleomycin.

MATERIALS AND METHODS

Patients and Evaluation Procedures

From June 1970 to June 1971, at the National Cancer Institute of Milan, 192 patients (148 men and 44 women) with different forms of solid tumors were treated with bleomycin for phase I and II evaluation. The average age was 55 years (minimum, 7 years; maximum, 83 years). Of 192 cases entered in the study, 168 were suitable for the evaluation of acute and delayed toxicity (table 1).

All patients had far-advanced neoplastic disease with a life expectancy of approximately 3–4 months. Most patients were resistant to conventional chemotherapy. The relatives of

TABLE 1.—Dose schedules of bleomycin and cases evaluable for acute and delayed toxicity

No. of patients entered in study	No. of evaluable cases	Dose schedule*	Total dose (mg/m^2)
25	18	A: 30 mg/m^2 given twice weekly for 4 wks. Course repeated after 1-mo interval.	480 in 13 wks
20	18	B: 30 mg/m^2 given for 8 consecutive days. Course repeated after 1-mo interval.	480 in 8 wks
33	31	C: 15 mg/m^2 given for 8 consecutive days. Course repeated after 1-mo interval.	240 in 8 wks
81	70	D: 15 mg/m^2 given for 5 consecutive days. Course repeated twice at 3-wk intervals.	225 in 11 wks
26	26	E: 15 mg/m^2 given twice weekly for 4 wks. Course repeated after 1-mo interval.	240 in 12 wks
7	5	F: 10 mg/m^2 given twice weekly for 4 wks. Course repeated after 1-mo interval.	160 in 12 wks
192	168		

*Maintenance treatment (with the initial dose per square meter of body surface area) was given once a week after a 1-mo interval.

the patients were informed of the potential hazard of pulmonary toxicity.

Blood chemistry tests, complete blood cell counts, and chest roentgenograms were obtained for all patients before drug administration. During the treatment and observation periods, chest roentgenograms were performed every 12–15 days and, occasionally, more often, when pulmonary toxicity was evident or suspected. Blood cell counts and blood chemistry tests were also repeated periodically. Pulmonary function tests were not carried out during this study.

Drug Administration

Bleomycin hydrochloride, available as a whitish dry powder in 15-mg ampules, was diluted in 0.9% NaCl and administered via a rapid single iv injection. The doses and schedules used during this trial are reported in table 1.

Because of the reports of relatively low incidences of pulmonary complications (ranging from 3.3% to 4.2%, as reported by the European Organization for Research on the Treatment of Cancer [EORTC] [4] and the largest Japanese study [23] respectively), during the first part of the trial high doses were administered in an attempt to achieve a higher percent of consistent and prolonged regressions in selected types of tumors (carcinoma of the head and neck, epidermoid carcinoma of the lung, and malignant lymphoma).

Due to the increasing occurrence of pulmonary damage during our study, it was decided not to expose the patients to high doses of bleomycin within a relatively short period of time. Therefore, as reported in detail in another paper (7), for the first four dose schedules (A, B, C, and D) (*see* table 1 for an outline of schedules) only about half the patients completed the entire course as originally planned. Moreover, to further minimize the occurrence of delayed toxicity, bleomycin therapy was stopped for 3–4 weeks (schedules E and F) at the first appearance of one of the initial side effects (skin changes, alopecia, or stomatitis). During this study an adequate dose of bleomycin was considered that which induced one or more signs of toxicity.

Once pulmonary toxic signs and symptoms were evident or merely suspected, bleomycin administration was stopped and prednisone or prednisolone [5] (50 mg/day) as well as antibiotics were given for about 2 weeks.

RESULTS: EARLY TOXICITY

Table 2 reports the incidence of all toxic manifestations occurring usually within the first 4 weeks after the administration of bleomycin, regardless of the total dose and the schedule. While schedules C and D reduced the degree rather than the frequency of side effects, schedules E and F produced stomatitis in only 25% of the patients and alopecia in 40%. Aside from pyrexia, nausea, and vomiting, with all dose schedules the first sign of toxicity occurred in most patients after a dose of 60–75 mg/m² (mean minimal toxic dose). With the exception of the hyperpigmented stria located preferentially throughout the back, all side effects were reversible.

RESULTS: DELAYED TOXICITY

Definition

Delayed toxicity was defined in the present trial as toxicity occurring usually after the first month of therapy. It consisted almost always of pulmonary damage.

[5] Prednisone: NSC–10023; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.
Prednisolone: NSC–9900; pregna-1,4-diene-3,20-dione, 11β,17,21-trihydroxy-.

TABLE 2.—Incidence of side effects occurring in 168 patients with evaluable cases during the first month of treatment with bleomycin

Signs of toxicity	Occurrence (%)
Sclerotic changes of the skin of hands	88
Skin hyperpigmentation	79
Alopecia	68
Fever	70
Stomatitis	47
Gastrointestinal disturbances	26
Generalized erythema	9

Incidence

Lung toxicity occurred in 67 of 168 patients with evaluable cases (39.8%). In 31 patients (46%), it was established using physical criteria and in 36 patients (54%) using both physical and radiologic criteria. In seven patients (4.2%), it was detected also at postmortem examination.

The incidence of pulmonary damage appeared to be slightly higher in patients older than 50 years, but no clear-cut relation could be found between the age of the patients and the total dose of bleomycin causing pulmonary toxicity (7). Also, the incidence of drug-induced pulmonary lesions was not significantly higher in the patients whose chest roentgenograms were normal and those with either chronic pulmonary disease or with neoplastic lesions in the lung (7). As reported in table 3, with the first three schedules the incidence of pulmonary toxicity ranged from approximately 67% to about 50% of the patients. With schedules D and E the incidence was reduced to 33% and 27% respectively. At the time of this writing, no patients treated with schedule F have showed signs of lung damage. Lung toxicity occurred after the administration of doses less than 100 mg/m² (minimum, 75 mg/m²), as detected at autopsy. Symptoms appeared usually after 4–10 weeks of therapy.

Physical Findings

Pulmonary toxicity was defined in clinical terms as the occurrence of rales, ronchi, and, occasionally, pleural friction rubs in patients without clear-cut signs of lung infection, congestive heart failure, or progression of neoplastic disease in the lungs. The initial physical findings consisted in a shower of fine crepitant rales which could be heard at the base of one or both lungs. If drug administration was stopped promptly, rales gradually regressed and disappeared within about 2 months. The detection of crepitations was followed within 1–2 weeks by early radiologic signs of interstitial involvement in about half the patients (54%). On the contrary, in the remaining patients (46%) typical roentgenographic findings were not present, probably because the lesions remained at a very early stage. In some cases, however, the radiologist concluded that these signs were compatible with acute bronchitis. Pulmonary toxic signs may progress if not detected at their onset or if high doses of bleomycin have been administered. In a few patients, however, progression occurred even after early diagnosis and after minimal total doses of bleomycin had been given. Auscultation of the lungs initially revealed subcrepitant rales which became gradually, but sometimes rapidly, moist and coarse, and spread throughout the lower two thirds of the thoracic cage. Ronchi could be heard also, mixed with rales. Occasionally, the typical creaking noise of the pleural friction rub was detected at one or both bases. In more advanced stages, bronchopneumonia was often accompanied by fever, cough, and dyspnea; signs and symptoms then progressed inexorably into acute respiratory failure despite intensive supportive care.

TABLE 3.—Breakdown of pulmonary toxicity in relation to the 6 dose schedules of bleomycin and the total dose (67 patients)

Dose schedule*	Toxicity		Mean total toxic dose (mg/m²)	Onset of toxicity (days)	
	No. of patients/ No. of evaluable cases	Occurrence (%)		After start of therapy	After last dose
A	12/18	66	359	71	10
B	10/18	55	283	55	8
C	15/31	48	135	70	17
D	23/70	33	132	50	9
E	7/26	27	93	28	13
F	—/5	0	—	—	—

*See table 1 for definitions.

Radiologic Observations

Pulmonary toxicity was defined using radiologic methods as the appearance of interstitial lesions. In cases of this nature, the initial diagnosis may not always be clear cut and should be performed by an experienced physician. Serial chest roentgenograms and strict correlation with physical observations are also required. During the first part of our phase I study, pulmonary toxicity was somewhat overlooked both clinically and radiologically, and the diagnosis was made only retrospectively. Therefore, ten patients received an additional dose of bleomycin while lung damage was actually already present (5).

Early pulmonary damage occurred as a fine reticular and reticulomicronodular pattern located usually at the lower lung zones and at the level of costophrenic angle (figs 1–3). The initial roentgenographic findings could also be detected in areas of previous chronic pulmonary diseases (emphysema, tuberculosis, etc).

The initial signs could evolve into a coarse streaking reticulation (fig 4). As for physical signs, after prompt discontinuation of bleomycin therapy and administration of corticosteroids plus antibiotics, a gradual regression to a complete roentgenographic resolution could be observed within 1–4 months (fig 5). In more advanced stages, the reticulomicronodularity became widespread and confused with patchy infiltrates (fig 6). These infiltrates were probably due to a superimposed bronchopneumonia. Regressions could be seen also at this stage of evolution. No definite radiologic signs of pulmonary fibrosis have been observed at this time, probably due to the still relatively short period of observation (maximum, 12 months).

The pulmonary lesions were never associated with mediastinal or hilar lymph node involvement. Pleural effusion occurred rarely. As al-

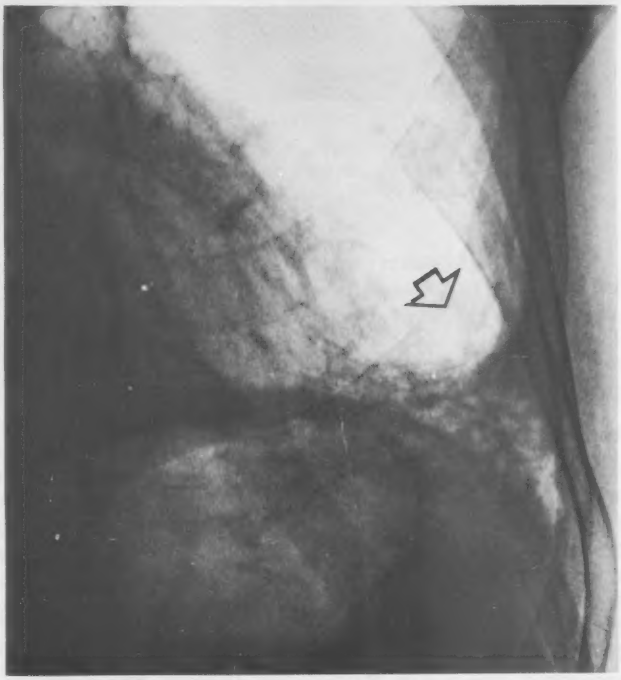

FIGURE 1.—Posteroanterior roentgenogram with magnified view of the left costrophrenic angle. Shows reticulomicronodularity which is typical of early bleomycin damage.

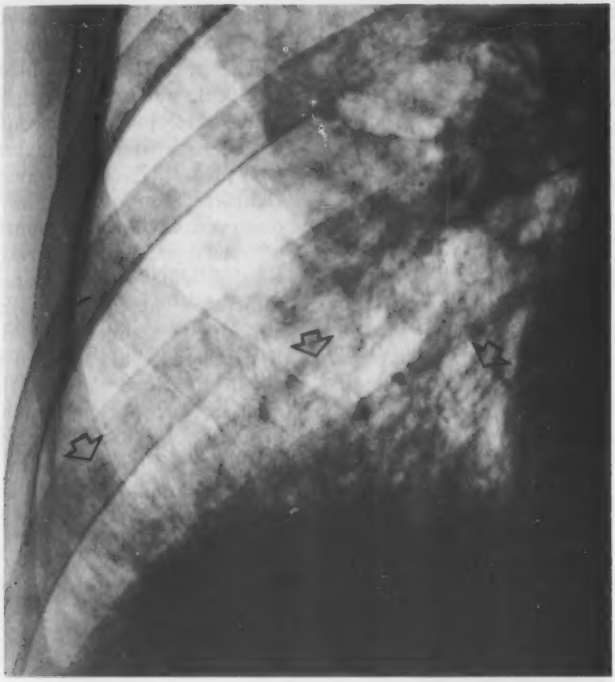

FIGURE 2.—Posteroanterior roentgenogram with magnified view of the right lung base. Shows fine interstitial reticulation and reticulonodularity above the diaphragm which occur in early pulmonary toxicity.

ready mentioned, the early roentgenographic observations were usually made 1–2 weeks after the occurrence of crepitant rales. In four patients, however, clear-cut signs of interstitial lesions were observed in complete absence of concomitant physical signs.

Histopathologic Observations

Postmortem examination was performed in 11 of 15 patients who died in the hospital. No significant lesions attributable to bleomycin therapy were observed on gross examination. Histologic sections showed a polymorphic picture, with different stages of evolution often being present within the same lung (24). No preferential distribution of lesions among the different pulmonary zones was seen. Early lesions were characterized by edema of the alveolar wall and the interstitial tissues. The alveolar space appeared to be filled by fibrinous edema (fig 7). Endoalveolar hyaline membranes were noted in two patients. Hyperplasia and endoalveolar migration of the type II pneumocytes and macrophages as well as proliferation of reticuloendothelial cells were observed in all patients (fig 8). In more advanced stages newly formed reticular and collagen fibers appeared at the level of basal membranes and within the alveolar septa (fig 9). Hyperplasia of the muscular fibers of respiratory bronchioli (24) and squamous metaplasia of the bronchiolar epithelium were also noted (8,9,11).

The pulmonary pattern became gradually replaced by fibrous tissue (fig 10). The histologic observations were consistent with the diagnosis of "sui generis" interstitial pneumonia (24). The lesions were focal or diffuse. The diagnosis could be difficult if previous chronic pulmonary damage or extensive neoplastic lesions or both were present.

FIGURE 3.—Posteroanterior roentgenogram showing diffuse reticular pattern mostly at the base of the left lung after therapy with bleomycin.

Implications in the Presumptive Cause of Death

Followup studies of patients who had pulmonary toxic reactions are reported in table 4. For the sake of simplicity, the physical and radiologic observations have been summarized as regression, no change, and progression at the time of this writing.

In the first group of patients, in whom pulmonary toxic effects were detected by physical diagnosis only, seven patients were lost to followup. Rales completely disappeared in eight patients, remained unchanged in 11, and progressed in terms of degree and extent in five. These findings occurred in 10, 13, and 13 patients of the second group respectively (radiologic or physical plus radiologic diagnosis).

TABLE 4.—Course of pulmonary toxicity in 67 patients treated with bleomycin and presumptive cause of death (26 patients)

Method of diagnosis of toxicity	No. of patients with toxic reactions	Followup study		Cause of death			
				Clinical*		Anatomic† (postmortem examination)	
		Results	No. of patients	Cause	No. of patients	Cause	No. of patients
Physical criteria only	31 (46%)	Lost to followup	7				
		Regression	8				
		No change	11	Cancer	3		
		Progression	5	Cancer + bleomycin	6		
Radiologic or physical plus radiologic criteria	36 (54%)	Regression	10				
		No change	13	Cancer	6 (4)	Cancer	4
		Progression	13	Cancer + bleomycin	6 (1)	Cancer + bleomycin	1
				Silicosis + bleomycin	1 (1)	Silicosis + bleomycin	1
				Bleomycin	3		
				Bleomycin	1 (1)	Bleomycin	1
					26		7

*Numbers in parentheses indicate the number of patients in whom postmortem examination was carried out.
†Four other patients died of progressive cancer without physical or radiologic signs of pulmonary toxicity. Postmortem examination confirmed the absence of drug-induced damage.

A total of 26 of 67 patients died within 2–8 weeks after the diagnosis of pulmonary toxicity. No deaths occurred among the 18 patients in whom pulmonary toxic signs showed regression. In the first group of patients (physical diagnosis), five of nine patients died at home, making the presumptive cause of death more difficult to establish. However, although these patients had progressive primary or secondary neoplastic lesions in the lungs, we cannot exclude the possibility that bleomycin might have contributed to a certain degree to the cause of death. In the second group of patients (radiologic or physical plus radiologic diagnosis), the presumptive cause of death could be established in seven instances by a postmortem examination. In all of these cases the histologic sections of the lungs revealed different grades of lesions attributable to bleomycin toxicity. In particular, they were focal in four patients and diffuse in three. From an anatomic point of view, we have evidence that only one patient died because of the direct action of bleomycin. In all other cases, both clinical and histopathologic diagnosis did not indicate a predominant action of the drug as far as the cause of death is concerned. Bleomycin, however, must have contributed somewhat to the death of ten other patients, six of whom also had extensive pulmonary neoplastic lesions and one of whom had widespread silicosis. Therefore, the mortality rate attributable to pulmonary toxicity could be estimated to be about 6.5%.

DISCUSSION

Several agents are known to produce pulmonary damage after the administration of different doses and within different periods of time. Lung injury occurs after the injection of some toxic organic compounds, such as herbisides and kerosene-base furniture polish, as well as the administration of drugs such as nitrofurantoin, diphenylhydantoin, hexamethonium chloride, mecamylamine, para-aminosalicylic

FIGURE 4.—Posteroanterior roentgenogram showing diffuse, coarse, streaking, interstitial lesions.

acid, sulphadimethoxine, procainamide, hydrochlorthiazide, and methysergide (25,26). Among the anticancer drugs, a folic acid antagonist (methotrexate) (27,28) and an alkylating agent (busulfan) (29–33) have been reported to produce pulmonary changes in a num-

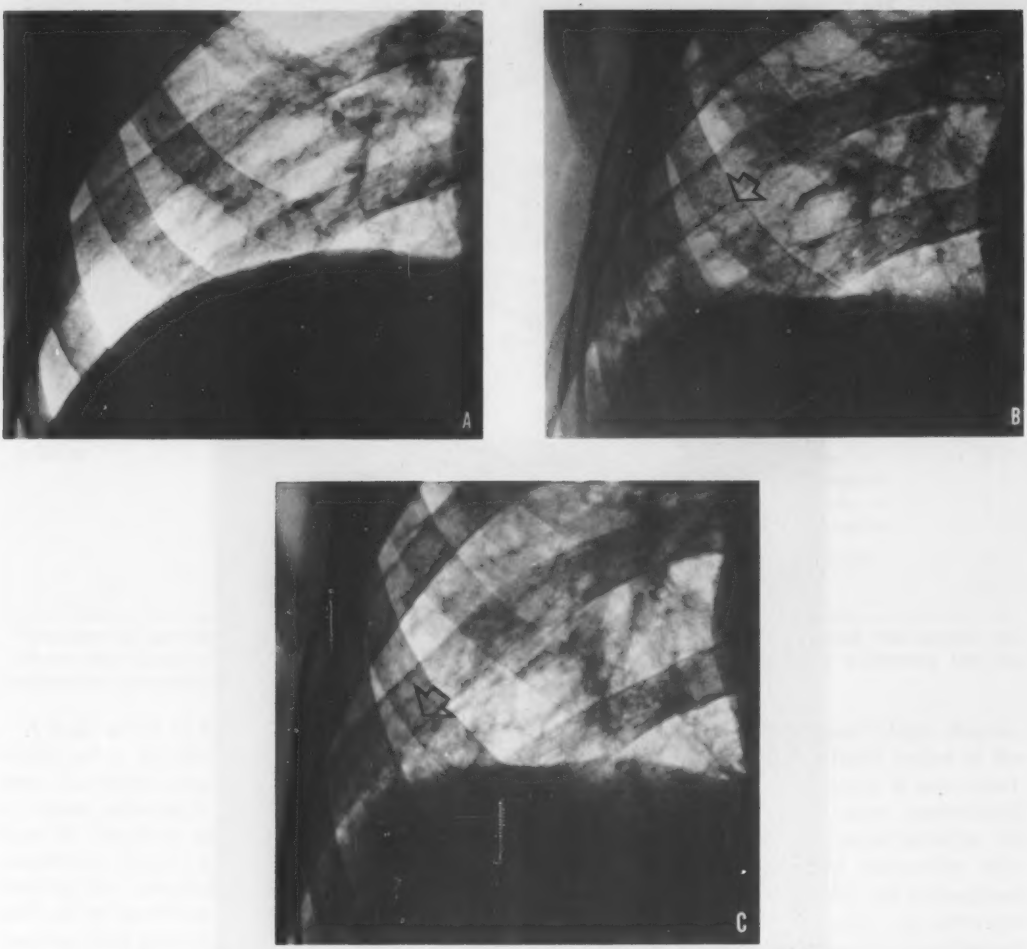

FIGURE 5.—Posteroanterior roentgenogram with magnified view of the right lung base. (A) Normal appearance before therapy. (B) Fine and coarse interstitial reticulonodularity after administration of bleomycin. (C) Marked regression of lesion about 2 months after discontinuation of bleomycin and administration of corticosteroids.

ber of patients.[6] The mechanism by which these drugs produce pulmonary disease is unknown, but a hypersensitivity reaction is probable (25,26,31). From a radiologic point of view, the lesions appear either as acute and often diffuse alveolar or interstitial lesions with or without the signs of hypersensitivity, or as chronic interstitial infiltrates, usually more marked at the bases (26).

These adverse drug reactions usually do not leave residual changes, with the exception of those produced by busulfan. The changes induced by busulfan are represented by atypical bronchial and alveolar cells, fibrinous edema, hyaline membranes, and intra-alveolar and interstitial fibrosis (29,30). Dysplastic changes

[6] Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2, 4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Busulfan: NSC-750; 1,4-butanediol, dimethanesulfonate.

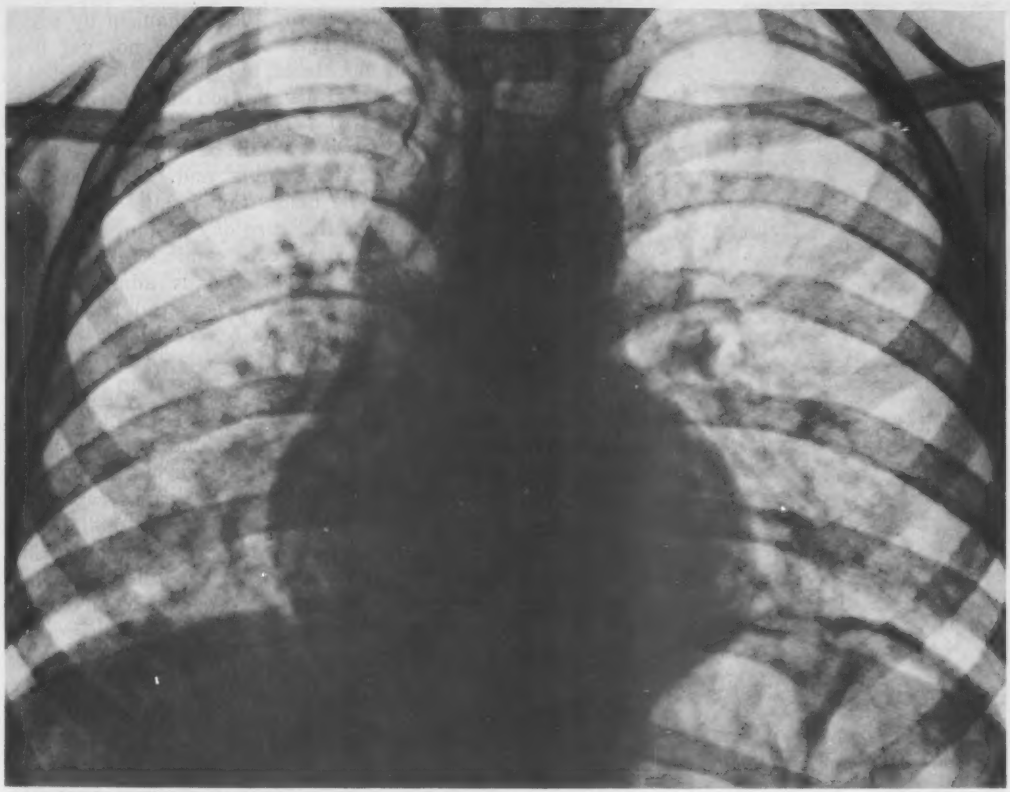

FIGURE 6.—Posteroanterior roentgenogram of a 7-year-old boy showing diffuse interstitial lesions mixed with patchy infiltrates.

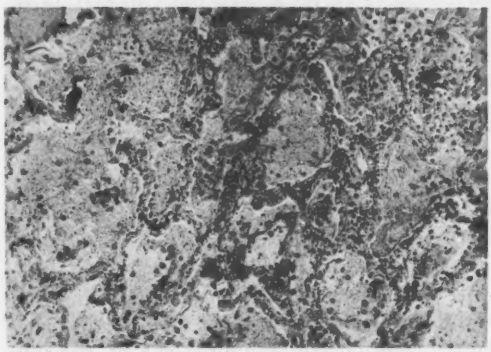

FIGURE 7.—Photomicrograph of the lung showing fibrinous edema (hematoxylin-eosin stain, × 550).

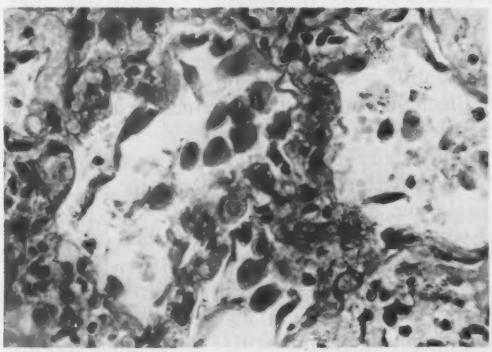

FIGURE 8.—Photomicrograph of the lung showing atypical alveolar cells (hematoxylin-eosin stain, × 550).

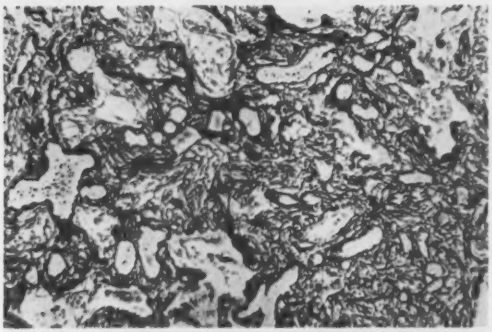

FIGURE 9.—Photomicrograph of the lung showing marked increase of reticular fibers (silver stain, × 120).

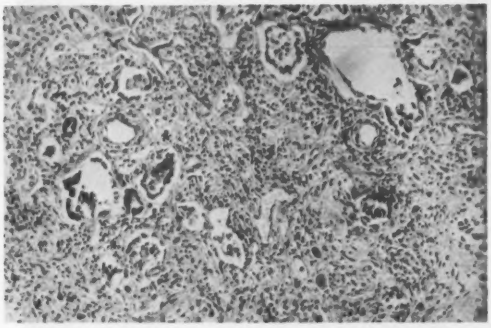

FIGURE 10.—Photomicrograph of the lung. Normal pulmonary pattern is replaced by fibrous tissue (hematoxylin-eosin stain, × 120).

in the epithelial cells of various organs were reported by several authors (30,33) and they are similar to those associated with methotrexate therapy (28); however, the lung toxicity induced by busulfan is rare.

Bleomycin-induced pulmonary damage is characterized clinically by crepitant rales, radiologically by reticular and reticulomicronodular patterns, and histologically by hyperplasia and endoalveolar migration of the type II pneumocytes and macrophages, fibrinous edema, hyaline membranes, and newly formed reticular and collagen fibers within the alveolar septa. These observations are consistent with the diagnosis of interstitial pneumonia and are in keeping with those of Japanese (12,14,22,34) and American (8,9,11) investigators.

The nature of and the mechanism by which pulmonary changes occur have not yet been completely elucidated, but they can be related to the preferential distribution of the drug in the lung tissue (21). Therefore, the incidence of pulmonary toxicity can be enhanced by administering high single and total doses of bleomycin. The disposition studies (21) show also that after the third day of administration, bleomycin is practically undetectable in the urine. Hence, prolonged daily administration of bleomycin is another potential hazard, resulting in a high rate of occurrence of lung toxicity (5–7,9).

Interstitial pneumonia with fibrosis represents an undesirable drug effect which is due to the pharmacologic action and, probably, to inappropriate dosage of bleomycin. Because of the dose-related pulmonary toxicity, patients receiving a total dose greater than 100–150 mg/m^2 within a relatively short period of time should be followed very carefully to detect early signs of pulmonary damage. Our experience proves that the initial signs of pulmonary toxicity can be detected by a systematic use of the stethoscope.

Early detection appears to be extremely important since initial lesions can regress completely (clinically and radiologically) provided bleomycin administration is stopped promptly. The recognition of bleomycin pulmonary toxicity may at times be difficult and requires accuracy, experience, and long-term followup. This is probably why the incidence of pulmonary damage varies among the different Japanese reports (12,14,22,23,34,35) and the EORTC (4), despite the fact that all groups used approximately the same drug regimen. Ichikawa (23) reported the development of lung fibrosis in 4.2% and pneumonia in 3.4% of 462 patients who had squamous cell carcinoma of various organs. Mathé et al (4) noted a pneumonia-like shadow in 3.3% of 237 patients who had different types of tumors, and Kimura (35) noted this reaction in 12% of 67 patients with carcinoma of the lung and malignant lymphomas. Lung biopsies and systematic postmortem examinations of patients who died after treatment yielded a higher incidence of drug-induced pulmonary injury. Oka

et al (12) observed histopathologic signs of lung toxicity in 13.7% of the lung biopsies of 29 patients with lung cancer. Arai et al (22) observed signs in 42% of seven patients with pulmonary carcinoma, Suzuki et al (14) observed signs in 50% of 24 patients with carcinoma of the esophagus, and Tamura et al (34) observed signs in 83% of six patients with cancer of the lung. The findings of Luce et al (9) are similar to those we observed with our first three schedules since these investigators used comparable single and total doses of bleomycin.

In conclusion, pulmonary toxicity induced by bleomycin represents a new and peculiar aspect of iatrogenic pathology. Its actual incidence is not yet established, either because different dose schedules have been used in phase I studies in Europe and in the United States, or because lung biopsies and autopsies have not been systematically performed in the early Japanese studies. Our observations support the hypothesis that this adverse drug reaction occurs more frequently than previously reported, although a larger number of patients treated with our schedule F (similar to the Japanese regimen) are required to confirm this contention. The occurrence of lung toxicity, although dose dependent, is at times unpredictable, and can be observed even after very small total doses (7,8). In our experience, as well as in that of some Japanese authors (35), no clear-cut relation could be found between the age of the patients and previous lung disease. Early detection is essential to avoid irreversible respiratory insufficiency. The therapeutic effects of bleomycin, although promising in the treatment of malignant lymphomas and some epidermoid carcinomas (5–9), appear to be short-lived (0.5–2.0 months), with or without maintenance treatment (7,9). Furthermore, the drug has no consistent toxic effect on the bone marrow precursors.

Bleomycin, therefore, appears to be the ideal drug to be administered in combination with myelosuppressive agents in future trials. Short intermittent courses with small doses are expected to decrease the incidence of pulmonary toxicity.

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Treatment of Metastatic Malignant Melanoma With a Combination of 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), Cyclophosphamide (NSC-26271), and Vincristine (NSC-67574) [1,2,3]

S. Gardere, S. Hussain, and D. H. Cowan [4,5]

SUMMARY

Courses of treatment with a combination of 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (NSC-45388) (850 mg/m²), cyclophosphamide (750 mg/m²), and vincristine (2.0 mg/m²) were given intravenously in single doses to patients with metastatic malignant melanoma. Treatment was repeated at 4-week intervals. Objective responses were observed in five of 20 patients (25%), indicating that treatment with this drug combination is at least as effective as treatment with NSC-45388 alone in either 5-day courses or intermittent, high-dose, single injections. The toxicity resulting from the combined treatment, although somewhat greater than that reported with the 5-day courses of NSC-45388, is acceptable and much less severe than that found with the single, high-dose schedule. Although the combination does not appear to increase the tumor response rate, it does maintain the advantage of a brief hospitalization for the patient.

[Cancer Chemother Rep 56:357-361, 1972]

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388; DIC) is the most effective agent now available for the treatment of metastatic malignant melanoma. When the drug was administered at a dose of 200–250 mg/m²/day for 5 days, at 3-week intervals, approximately 20% of the treated patients experienced a useful remission (1). We reported a similar remission rate for NSC-45388 administered in single doses (650–1450 mg/m²) at 4-week intervals (2). Drug toxicity was excessive with doses of 1050 mg/m² or greater, but was quite acceptable with doses of less than 950 mg/m². Because an effective treatment that requires brief hospitalization is advantageous, we decided to explore the use of other drugs in combination with single injections of NSC-45388 at a dose less than 950 mg/m². Cyclophosphamide and vincristine were chosen because their patterns of toxicity differ and because both drugs had caused tumor regression in some patients with metastatic melanoma (3,4).

[1] Received Sept 29, 1971; accepted Jan 26, 1972.

[2] Supported by The Ontario Cancer Treatment and Research Foundation, Toronto, Canada.

[3] Cyclophosphamide: 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Vincristine: leurocristine, sulfate (1:1).

NSC-45388 was supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, National Cancer Institute.

[4] Department of Medicine, University of Toronto, and The Ontario Cancer Institute and the Princess Margaret Hospital, Toronto, Canada.

[5] *Reprint requests to:* Dr. D. H. Cowan, 500 Sherbourne Street, Toronto 5, Ontario, Canada.

MATERIALS AND METHODS

Patient Selection

Twenty patients with malignant melanoma and metastatic lesions that could be measured

by either clinical or radiographic means were accepted for the study. Eight patients had received radiotherapy to local lesions from 2½ to 20 weeks prior to chemotherapy. One patient received a single dose of irradiation to the brain with each of three courses of chemotherapy. The same patient also received dexamethasone during the study period.[6] In all of these patients, lesions which had not been treated with radiotherapy were present and could serve as indicators of response to the drugs. One patient had received melphalan by intra-arterial perfusion 8 weeks before the study, and one patient had received systemic treatment with thioTEPA 12 weeks before the study.[7]

Drug Administration and Dose Schedule

After a preliminary study in five patients to determine an acceptable dose schedule, the following protocol was used: NSC-45388 at a dose of 850 mg/m², cyclophosphamide at a dose of 750 mg/m², and vincristine at a dose of 2.0 mg/m². The three drugs were administered sequentially, by the intravenous (iv) route, over a total of 10–15 minutes. Repeat courses were given at 4-week intervals unless the patient had not fully recovered from the toxic effects of treatment. Doses were decreased appropriately if excessive toxicity occurred. Premedication with prochlorperazine (Stemetil), diphenoxylate atropine (Lomotil), and sodium amytal was used to control vomiting and diarrhea. After the drugs had been given, iv solution was continued overnight; all patients were hospitalized for at least 24 hours. An attempt was made to give at least three courses of therapy, but treatment was stopped if there was evidence of rapid tumor progression.

Case Evaluation and Followup

The complete medical history of each patient and the results of physical examination were evaluated. Performance status (5) was recorded for each patient. The diameters of measurable lesions were determined in two perpendicular planes. Pretreatment laboratory investigations included: (*a*) determination of hemoglobin level, leukocyte count and differential, and platelet and reticulocyte counts; (*b*) determination of blood urea nitrogen (BUN), serum creatinine, serum alkaline phosphatase, and serum glutamic oxaloacetic transaminase (SGOT) levels, and sulfobromophthalein (BSP) test; (*c*) chest roentgenogram and skeletal survey; (*d*) tests for urine melanin; and (*e*) other investigations as indicated.

Followup clinical and laboratory investigations included: (*a*) complete physical examination with measurements of lesions at 4-week intervals; (*b*) determination of hemoglobin level, leukocyte count and differential, and platelet and reticulocyte counts twice weekly; (*c*) determination of BUN, SGOT, and serum alkaline phosphatase levels once weekly; and (*d*) other investigations as indicated.

Assessment of Response

A partial remission was defined as a greater than 50% reduction in measurable tumor provided no deterioration of the patient's condition had occurred and no new lesions had appeared; a complete remission was defined as the disappearance of all subjective and objective evidence of disease.

RESULTS

Twenty patients received a total of 58 courses of therapy. The number of courses administered to patients and the performance status of each patient at the beginning of chemotherapy are summarized in table 1.

Twelve patients received only one or two courses of treatment. Three patients died of their disease within 3 weeks of the first course (initial performance status, 50, 20, and 20), and treatment was not continued in the remaining nine patients because they had rapidly progressing disease.

[6] Dexamethasone: NSC-34521; pregna-1,4-diene-3,20-dione, 9-fluoro-11β,17,21-trihydroxy-16α-methyl-.

[7] Melphalan: NSC-8806; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-phenylalanine mustard.

ThioTEPA: NSC-6396; phosphine sulfide, tris(1-aziridinyl)-; triethylene-thiophosphoramide.

TABLE 1.—Number of courses of chemotherapy administered and performance status of patients treated

No. of courses	No. of patients	Performance status
3–12	8	90,90,90,90,90,80,50,30
2	5	90,90,60,50,20
1	7	90,90,80,70,40,20,20

Antitumor Effect

Five patients had significant responses to antitumor treatment. The data concerning these patients are summarized in table 2. For each of these patients, there was definite evidence of tumor regression after the first course of treatment. Although the performance status was 80 or greater in three of these individuals, one patient (No. 3) was extremely ill with a performance status of 30. The remarkable, although brief, improvement in this patient encouraged us to enter the several other patients with performance status less than 40 into the study.

Three additional patients experienced some tumor regression which was associated with progression of disease in other areas. In the entire group, regression was observed in metastases involving the skin and subcutaneous tissues, lymph nodes, lungs, liver, and spleen, but not in metastatic tumors of the brain or bone tissue.

TABLE 2.—Pretreatment assessment of disease and results of combined treatment (NSC-45388, vincristine, and cyclophosphamide) in responding patients

Patient No. (age, sex)	Previous treatment	Pretreatment assessment		No. of courses of combined treatment	Response
		Performance status	Organ involvement		
1 (33,M)	Radiotherapy to neck mass 2 wks before study	90	Lymph node, spleen	12	Partial remission for 37 wks; performance status remained 90; all lymph nodes disappeared; spleen impalpable but results of scan remained abnormal; relapse with recurrent lymph node enlargement
2 (45,M)	None	80	Multiple skin nodules	4	Complete remission for 12 wks; performance status 100; gained 4.5 kg; relapse with skin nodule and spinal cord compression
3 (43,F)	Melphalan infusion (left leg) 2 mos before study	30	Liver, lungs, pleura	4	Partial remission for 9 wks; performance status 90; gained 5 kg; relapse with regrowth of pulmonary metastasis
4 (62,M)	Removal of single cerebral metastasis 5 mos before study; radiotherapy to rib 1 mo before study	90	Multiple skin nodules, lungs, bone	7	Partial remission for 29 wks; performance status 90; hemoglobin level rose from 9.0 to 12.0 g/100 ml; relapse with recurrence of intracerebral metastasis
5 (45,F)	Removal of cerebral metastases 11 mos before study	50	Brain, lymph node, skin, lung	5*	Partial remission for 12 wks; performance status 70; relapse with regrowth of lymph node

*This patient received dexamethasone in oral doses throughout the period of study and a single dose of 500 rads radiation to the brain with the first three courses of chemotherapy.

Toxicity

General.—All patients but one experienced nausea and vomiting after at least one course of treatment. The nausea and vomiting usually started within 3 hours of drug administration and lasted 1–24 hours. In many patients, these symptoms were less severe with subsequent courses. Twelve patients also had diarrhea which lasted for several hours after treatment. The diarrhea was less severe than that which had been observed with higher doses of NSC-45388 (2). Several patients experienced fever with temperatures up to 39.5 C which lasted 3–6 hours after drug administration. Significant alopecia developed in nine patients. A delayed "flu-like" syndrome, with abdominal pain, weakness, nausea, and occasionally, fever, was observed in six patients, but again, these symptoms were less frequent and less severe than those observed in our previous study. Vincristine neurotoxicity, necessitating a reduction in dose or omission of the drug, occurred in 11 patients. We had the impression that this side effect was more marked with this combination therapy than with vincristine given alone at the same dose. There was a transient and mild elevation of SGOT level in seven patients, of serum alkaline phosphatase level in two patients, and of BUN level in six patients. These values returned to normal before the next course of treatment.

Hematologic.—Hematologic toxicity could be evaluated for 55 of the 58 courses in 17 of the 20 patients who were treated. The major effects were neutropenia and thrombocytopenia. There was no bleeding and no recognized infection. The occurrence and timing of granulocyte and platelet count depression and recovery are shown in table 3. It is of interest that the nadir and recovery occurred earlier with combined treatment than with NSC-45388 given alone in a single injection (2) and were closer in timing to that which would be expected with high-dose intermittent cyclophosphamide treatment (6).

DISCUSSION AND CONCLUSIONS

Five of 20 patients (25%) had significant remissions. Although comparison of different groups of patients treated at different centers or at different times is difficult, these results are comparable to those achieved with NSC-45388 given alone (1,2). Only eight of the 20 patients received what was initially considered to be an adequate trial (three courses) and, of these eight patients, five responded. However, for all patients who responded, there was evidence of tumor regression after the first course of therapy. We interpret this to mean that patients for whom treatment was stopped after one or two courses because of rapid tumor progression suffered from an aggressive, unresponsive form of the disease. Of the 40 patients in this study and our previous study with NSC-45388, only one patient with an initial performance status of less than 50 responded. Furthermore, we have seen no regression of intracerebral or osseous metastases. Recognition of these factors should be helpful in selecting patients who are more likely to benefit from treatment. The neurotoxic effect of vin-

TABLE 3.—Granulocyte and platelet counts after chemotherapy

	No. of patients	No. of courses	Mean day of nadir ± 1 SD	Mean day of recovery ± 1 SD
Granulocyte count				
Nadir <1000 cells/mm³	10/17	24/55	14.9±2.46	20.6±2.35
Nadir <500 cells/mm³	7/17	10/55	14.0±2.39	19 ±3.53
Platelet count				
Nadir <150,000 cells/mm³	10/17	18/55	12.2±6.27	15.7±4.84
Nadir <100,000 cells/mm³	3/17	3/55	12 ±4.54	17 ±2.83

cristine given in combination with other drugs was not consistent with effects observed when the drug was used alone at a comparable dose. In the future, the dose of vincristine should be reduced to a maximum of 1.5 mg/m² when the drug is used in combination with NSC-45388. The toxicity resulting from the combination therapy was acceptable and less severe than that observed in the patients who received single injections of NSC-45388 alone at doses of 1050 mg/m² or greater.

In conclusion, this combination drug schedule does not improve the reported antitumor effect of NSC-45388 given alone in either repeated 5-day courses or intermittent single injections. The combination does, however, maintain the advantage over the 5-day schedule of brief hospitalization of the patient and less frequent visits to the outpatient department. It also maintains the antitumor effect of single high doses of NSC-45388, with the added advantage of less toxicity. The addition of cyclophosphamide and vincristine may not be beneficial, and it remains to be determined whether NSC-45388 given alone, in single injections of 850 mg/m², might not be as effective as any of the above schedules.

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Effective Combination Treatment of Advanced Acute Lymphocytic Leukemia With Cytosine Arabinoside (NSC-63878) and L-Asparaginase (NSC-109229) [1,2,3]

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SUMMARY

The combination of cytosine arabinoside and L-asparaginase induced remission in 15 of 22 children (68%) with previously treated acute lymphocytic leukemia (ALL). Eight of these patients were in remission after the second week of treatment and seven were in remission after the fourth week. The combination was well tolerated. These preliminary results suggest that these two agents have an additive therapeutic effect in inducing remissions in children with advanced ALL.
[Cancer Chemother Rep 56:363–368, 1972]

The ability to induce remissions in patients who have late refractory acute lymphocytic leukemia (ALL) is one criterion for identifying effective new agents or combinations of agents. In a series of clinical investigations conducted by the Children's Cancer Study Group A (CCSGA), cytosine arabinoside produced a remission rate of 30% when used alone (1) and 45% when used in combination with prednisone [8] in children with ALL refractory to conventional agents (2). L-Asparaginase has produced an overall remission rate of 44% in refractory patients (3). Because there is now abundant evidence (4,5) that agents with different mechanisms of action act additively when used in combination, a group of patients with acute leukemia whose diseases were refractory to conventional agents were treated with combinations of cytosine arabinoside and L-asparaginase. The observed remission rate was not significantly different from that calculated assuming independent drug action (4).

MATERIALS AND METHODS

Patients

Twenty-two consecutively admitted patients (17 from the Children's Hospital of Los Angeles, Calif, and five from the Children's Hospital of Columbus, Ohio) were given L-asparaginase (10,000 units/m^2 administered by a

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[2] Supported by Public Health Service grants CA–02649 (Children's Hospital of Los Angeles), CA–03750 (Children's Hospital of Columbus), and RR–00425 (Harbor General Hospital).

[3] Cytosine arabinoside: cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C. Drugs used in this study were supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, National Cancer Institute.

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[5] Dr. Karon: Scholar, Leukemia Society of America.

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[7] The secretarial assistance of Miss A. McBride and Mrs. A. Minhas is gratefully acknowledged.

[8] Prednisone: NSC-10023; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

30-minute intravenous [iv] infusion, three times a week) and cytosine arabinoside (90 mg/m² administered iv or intramuscularly [im] every day for 5 consecutive days per week) (table 1). These patients ranged in age from 1 to 16 years. All patients had more than 25% blast cells in the marrow aspirate at the start of treatment (table 1). Five patients had received cytosine arabinoside previously (Nos. 3, 10, 11, 13, and 18). Four patients had never attained remissions in spite of therapy with prednisone, vincristine, and methotrexate (Nos. 2, 4, and 5) and 6-mercaptopurine (6-MP) (No. 1).[9] Seven patients had never received 6-MP.

[9] Vincristine: NSC-67574; leurocristine, sulfate (1:1). Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-. 6-MP: NSC-755; purine-6-thiol, monohydrate.

TABLE 1.—Patient information

Patient No. (age, sex)	Wbc count at diagnosis (cells × 10^3/mm³)	Previous chemotherapy*	Wbc count on Day 0/nadir (cells × 10^3/mm³)	Best marrow status†	Days to best marrow	Result‡	Comments
1 (2 ¾, M)	9.6	Prednisone, vincristine, methotrexate, 6-MP, cyclophosphamide	2.3/1.3	1	14	CR	
2 (4 ⅓, M)	9	Prednisone, methotrexate, vincristine	6.0/2.1	1	28	CR	
3 (1, F)	100	Prednisone, vincristine, methotrexate, 6-MP, cyclophosphamide, cytosine arabinoside	3.0/0.5	1	14	CR	
4 (9, M)	59	Prednisone, vincristine, methotrexate	10.0/1.7	1	14	CR	
5 (6, M)	105	Prednisone, vincristine, methotrexate	13.3/1.2	1	28	CR	
6 (9½, M)	6.4	Prednisone, 6-MP, methotrexate, vincristine	5.8/0.1	3	—	NR	Anaphylactic reaction by Day 12
7 (2 ⅔, M)	3.9	Prednisone, 6-MP, methotrexate, vincristine, cyclophosphamide	3.2/0.6	1	28	CR	
8 (2 ⅙, M)	3.7	Prednisone, methotrexate, 6-MP, vincristine	9.1/1.0	3	—	IT	Died on Day 6 with massive intracranial bleeding
9 (4 ¾, F)	4.3	Prednisone, methotrexate, vincristine, cyclophosphamide	0.6/0.2	3	14	NR	Anaphylactic reaction by Day 14
10 (14 ¼, M)	21	Prednisone, methotrexate, 6-MP, vincristine, actinomycin D, cyclophosphamide, cytosine arabinoside	68.0/1.5	1	28	CR	
11 (16, F)	91	Prednisone, vincristine, 6-MP, methotrexate, cyclophosphamide, cytosine arabinoside	74.0/1.2	3	28	NR	

TABLE 1.—Patient information—Continued

Patient No.	Wbc count at diagnosis cells × 10^3/mm^3)	Previous chemotherapy*	Wbc count on Day 0/nadir (cells × 10^3/mm^3)	Best marrow status†	Days to best marrow	Result‡	Comments
12 (11, F)	51	Prednisone, methotrexate, vincristine, 6-MP	1.6/0.3	1	14	CR	
13 (15 7/12, F)	4.8	Prednisone, vincristine, methotrexate, 6-MP, cytosine arabinoside	—	3	—	IT	Died on Day 7 with streptococcal sepsis
14 (7 1/3, M)	227	Prednisone, vincristine, methotrexate, 6-MP	67.4/0.9	1	13	CR	
15 (3 1/3, M)	174	Prednisone, vincristine, methotrexate	10.9/2.9	2	14	PR	Marrow, 10% blast cells
16 (9, F)	77	Prednisone, vincristine, methotrexate	2.0/1.0	1	14	CR	
17 (16, F)	—	Prednisone, vincristine, methotrexate	4.3/2.7	1	14	CR	
18 (5, M)	62	Prednisone, vincristine, methotrexate, 6-MP, cyclophosphamide, cytosine arabinoside	3.0/0.6	3	23	NR	Died on Day 23 with *Escherichia coli* sepsis
19 (6, F)	3	Prednisone, methotrexate, 6-MP, vincristine, cytosine arabinoside	7.3/0.7	3	28	NR	
20 (6, F)	12	Prednisone, 6-MP, vincristine, methotrexate, cyclophosphamide	1.9/0.3	2	30	PR	
21 (8, M)	38	Prednisone, 6-MP, methotrexate, vincristine, cyclophosphamide	39.2/1.0	1	27	CR	
22 (3, F)	3	Prednisone, methotrexate, 6-MP, cyclophosphamide, actinomycin D, nitrogen mustard, vincristine	1.2/0.5	1	29	CR	

*Actinomycin D: NSC-3053.
Nitrogen mustard: NSC-762; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2.
Cytosine arabinoside: NSC-63878; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C.
†*See* table 2 for definitions.
‡CR = complete remission; PR = partial remission; NR = no response; IT = inadequate trial.

Bone marrow examinations were performed on Day 14 in 11 patients, and if the patient was still in relapse, treatment was continued for an additional 2 weeks. The remaining patients received treatment for 28 days before bone marrow examinations were performed. Since 77% of the patients (17 of 22) had white blood cell (wbc) counts of 1500 cells/mm^3 or less, the final dose of cytosine arabinoside was eventually reduced to 50 mg/m^2 (Nos. 15–17).

Evaluation of the extent of disease and the response to therapy was made according to criteria previously published by the CCSGA (6).

RESULTS

The results of this study are tabulated in table 1. Two patients died within 2 weeks of the start of therapy: one (No. 13) of sepsis on the seventh day of treatment, and the other (No. 8) of intracranial bleeding on the sixth day. Of the remaining 20 patients, eight achieved bone marrow and clinical remission by Day 14 and seven achieved such remission by Day 28 of treatment. Five patients failed to achieve remissions but in two of these, treatment had been discontinued after 12 and 14 days because of anaphylactic reactions to L-asparaginase. A third patient who failed to obtain a remission had previously received a 10-day course of cytosine arabinoside (5 mg/kg/day) combined with prednisone, to which he did not respond. Of four other patients who received previous treatment with cytosine arabinoside, two achieved a complete remission and one died of sepsis on Day 7. Previous treatment with cytosine arabinoside in this group consisted of a weekly injection of 5 mg/kg.

The cases of the 15 patients achieving remission were analyzed according to the quality of the bone marrow aspirate. Thirteen patients achieved completely normal bone marrow, and two had bone marrow blast cell counts of less than 10%. The overall remission rate was, therefore, 68% (15 of 22 × 100). The physical examination became completely normal in all patients who achieved M_1 and M_2 marrows according to the criteria of the CCSGA (table 2). If the two patients who died during the first week of therapy of causes unrelated to drug administration are excluded, the remission rate becomes 75% (15 of 20).

Seventeen of the 22 patients had wbc counts less than or equal to 1500 cells/mm³ (77%). The dose of cytosine arabinoside was reduced by 40%–60% in 15 of the 22 patients (68%) because of leukopenia. Three patients were given 50 mg/m² initially and in these patients wbc counts with nadirs of 1000, 2750, and 2900 cells/mm³ developed. They were able to tolerate the medication for 4 weeks. Two of these patients achieved complete remission and the other achieved good partial remission.

TABLE 2.—Bone marrow rating criteria for acute lymphocytic leukemia (CCSGA)

Parameter rating	Blast cells (%)	Lymphocytes + blast cells (%)
M_1	0–5	0–40
M_2	6–25	41–69
M_3	25	69

Only two patients had significant allergic reactions to L-asparaginase. One had wheezing, anxiety, and hypotension on Day 12 after six doses of the drug, and the other had vomiting, chills, hypotension, and fever on Day 14; neither obtained a remission. At the time of the reactions both patients had not been receiving cytosine arabinoside therapy because of leukopenia for 6 and 10 days respectively.

Of the 15 patients achieving remission, seven were given maintenance treatment with 6-MP; one of these patients had previously received 6-MP. Four other patients continued to receive cytosine arabinoside given im, and two patients received the oral form of this agent at a dose now known to be inadequate. One patient received a combination of cytosine arabinoside and cyclophosphamide [10] for maintenance therapy, and two received methotrexate given twice weekly orally. The median duration of remission for the 15 patients was 56 days with the longest remission lasting 199 days. Patients receiving 6-MP for the first time had the longest remissions. However, three of the patients receiving 6-MP for the first time had remission durations of less than the median.

Eight patients in this study had wbc counts in excess of 50,000 cells/mm³ at the time of diagnosis. Of these, seven achieved remission (six complete remissions, one partial remission). Of the three patients who had wbc counts in excess of 50,000 cells/mm³ at the time L-asparaginase and cytosine arabinoside treatment was started, two achieved remission and one did not.

[10] Cyclophosphamide: NSC-26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

DISCUSSION

Combinations of agents with different mechanisms of action and toxicities have been used to produce additive therapeutic effects. Such results have been obtained from all agents so used (5). The combination of cytosine arabinoside and L-asparaginase has been reported to induce remissions in mice with EARAD leukemia at a rate higher than when either drug was used alone (7). In the present study, 22 consecutively admitted patients were treated with a combination of cytosine arabinoside and L-asparaginase, and a remission rate of 68% was attained. In a study by the CCSGA, a larger group of patients refractory to conventional agents was treated with L-asparaginase alone, and a remission rate of 44% was attained (3). The difference between these two rates is significant, $P<0.05$. This high rate of remission induction in patients with ALL who are unresponsive to other agents is impressive, especially since four of these patients had never before achieved a remission while receiving conventional agents. In addition, 87% of the patients (seven of eight) whose initial wbc counts were 50,000 cells/mm³ or greater achieved remissions. This is a group of patients often thought to have an especially poor prognosis.

Using a different schedule, McElwain et al (8) obtained remissions in eight of nine patients with ALL who were treated with a course of cytosine arabinoside followed sequentially by two courses of L-asparaginase. It remains to be seen whether the high rate of remission in this smaller series of patients is significantly different from that obtained when both drugs were given simultaneously.

The relationship between the development of leukopenia in these patients and the successful induction of remission is of interest. Since leukopenia had been previously reported in only 34% of the patients treated with L-asparaginase alone at the same dosage level (3), the higher frequency of this complication in patients treated with the combination (77%) could be the result of an additive effect. The dosage of L-asparaginase was not modified because of the occurrence of leukopenia; the dosage of cytosine arabinoside, however, was modified. A study of this combination using a starting dose of cytosine arabinoside of 50 mg/m² is currently underway by the CCSGA.

Although cytosine arabinoside is known to be an immunosuppressant (9), the small number of patients in this study makes an evaluation of the ability of this drug to decrease the incidence of anaphylactic reactions difficult. 6-MP was found to reduce the incidence of anaphylactic reactions to this dose and schedule of L-asparaginase. In a study involving 160 patients, the incidence of anaphylactic reactions to L-asparaginase was 17% without the use of 6-MP and only 2% when L-asparaginase was given to patients also receiving therapy with 6-MP (10). Consequently, in our group of 22 patients, one would expect three patients to have had anaphylactic reactions in the absence of immunosuppression, and one or fewer if cytosine arabinoside was as effective as 6-MP. In fact, two patients had these reactions but only after cytosine arabinoside treatment had been discontinued.

No attempt was made to specify the maintenance treatment of those patients who achieved remissions. Not unexpectedly, the median duration of remission was longest for two patients who received maintenance treatment with 6-MP without having been previously treated with this drug, although none of the patients achieved the expected median remission of 6–8 months usually noted with 6-MP maintenance therapy. The median duration of remission for the entire group was short, 56 days. This is the length of an unmaintained remission in late refractory disease. L-Asparaginase maintenance therapy was not used since the enzyme has already been shown to be ineffective for maintaining remissions in late stages of the disease (8).

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Clinical Evaluation of 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), Melphalan (NSC-8806), and Hydroxyurea (NSC-32065) in the Treatment of Disseminated Malignant Melanoma [1,2]

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SUMMARY

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), melphalan, and hydroxyurea were evaluated in a randomized clinical trial in which 35 patients with disseminated malignant melanoma were treated. Therapeutic results were not impressive. Objective remissions were noted in two of the 23 patients who received NSC-45388, none of the 21 who received melphalan, and none of the 24 who received hydroxyurea. Toxicity was significant but tolerable: gastrointestinal toxicity was noted in 23 of the 23 patients who received NSC–45388, 15 of the 21 who received melphalan, and 16 of the 24 who received hydroxyurea. Hematologic toxicity resulted in leukocyte counts less than 3000 cells/mm^3 in seven of the 23 patients who received NSC-45388, six of the 21 who received melphalan, and 18 of the 24 who received hydroxyurea. Platelet depression, with cell counts less than 100,000 cells/mm^3, was noted in five of the 23 patients who received NSC-45388, three of the 21 who received melphalan, and six of the 24 who received hydroxyurea.

[Cancer Chemother Rep 56:369–372, 1972]

Many chemotherapeutic agents, including hydroxyurea (1), methotrexate, the vinca alkaloids, actinomycin D, various alkylators (2,3), and even hormones (4), have produced sporadic but unpredictable effects in the treatment of patients with disseminated malignant melanoma.[4] Few published studies, however, describe the evaluation of available compounds in prospective, randomized clinical trials.

5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), in its clinical trial phase, has consistently shown some promise in recent studies as an agent with more predictable activity in patients with malignant melanoma. Luce et al (5) reported an objective response in 19% of patients with malignant melanoma who were given NSC-45388 at a dose of 200–500 mg/m^2/day for 5 days, repeated at 3-week intervals. Recently, Nathanson and his colleagues (6) within the Eastern Cooperative Oncology Group reported a response in 28% of 115 patients with malignant melanoma for whom response was evaluable. In their study, a dose schedule of 2 mg/kg/day for 10 days proved more effectual than a dose schedule of 4.5 mg/kg/day for 10 days, repeated after 20 days, although the larger doses caused more toxic effects. Cowan and Bergsagel (7) recently reported responses in four of 20 patients with metastatic malignant melanoma who

[1] Received Oct 8, 1971; revised Jan 13, 1972.

[2] Melphalan:alanine, 3-[*p*-[bis(2-chloroethyl)amino]-phenyl]-, monohydrochloride, L-; L-phenylalanine mustard. The authors thank Dr. Stephen K. Carter, Chief, Cancer Therapy Evaluation Branch, Chemotherapy, National Cancer Institute, for his help and for supplying the drugs used in this study.

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[4] Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.
Actinomycin D: NSC-3053; dactinomycin.

were given NSC-45388. The patients in the series received NSC-45388 in single injections (at doses of 650–1450 mg/m^2) repeated at a median interval of 36 days. The responses observed indicated that the agent is not schedule-dependent.

In our study—a prospective, randomized clinical trial—NSC-45388 was compared with two other agents, melphalan and hydroxyurea, which had been reported previously as showing some activity in patients with malignant melanoma.

MATERIALS AND METHODS

All 35 patients in our series had disseminated (stage IV) malignant melanoma. Prior to randomization and treatment, the patients were grouped according to the presence or absence of visceral disease. Thirty of the 35 patients had visceral organ involvement, whereas five had disease limited to cutaneous or nodal dissemination. In these five patients, however, the metastatic lesions were scattered and were not amenable to surgical treatment. None of the patients had had previous chemotherapy with any of the drugs used in the study, and only four of the 35 had had previous chemotherapy of any kind. The patients were maintaining a reasonable state of nutrition and had not received radiation therapy to any significant extent. A frank discussion with the patient concerning the limitations and toxic effects of the proposed chemotherapeutic study was held prior to randomization, and all of the patients in the study had elected to participate. All of the patients had a hematologic status that did not preclude vigorous cytotoxic chemotherapy, that is, all had leukocyte counts greater than 4000 cells/mm^3 and platelet counts greater than 100,000 cells/mm^3.

After the extent of disease had been ascertained for patients in the study, the patients were randomly assigned to treatment with one of the three drugs under study. NSC-45388 was given intravenously (iv) at a dose of 275–400 mg/m^2/day for 5 days, with a mean daily dose of 340 mg/m^2. Hydroxyurea was given iv during a 5-hour period at a dose of 70–100 mg/kg/day for 5 days, with a mean daily dose of 86 mg/kg. Melphalan was given orally in divided doses over 5 days, to a total dose of 1.2 mg/kg.

After the treatment had been given, leukocyte counts and platelet counts were determined twice weekly for a 3-week period.

Each patient's status was re-evaluated 4–5 weeks after chemotherapy, at which time objective assessment of the drug effect was accomplished. Objective improvement was defined as a measurable reduction in the size of the indicator lesions by at least 33% of the product of the perpendicular diameters. Disease was considered progressive if any malignant lesions increased in size, regardless of the response of the remaining disease, or if new lesions developed during the interval period. Patients whose disease remained stable were retreated with the same agent until objective evidence of progressive disease appeared. For the most part, chemotherapy failed after the initial treatment.

When there was evidence of progressive disease, the patients were randomly allocated to receive one of the two remaining compounds being studied, and eventually, if further treatment could be tolerated, they received the third drug.

Of the 35 patients in the study, 19 were men and 16 were women. The median age was 45 years and the mean age was 47.5 years.

RESULTS

The therapeutic results were not impressive (table 1). The only drug that showed any objective evidence of antitumor effect was NSC-45388: two patients had objective evidence of tumor regression. The lengths of the remissions in these two patients were 8 and 2 months, respectively. Two other patients improved after receiving NSC-45388, but the reduction in the size of their indicator lesions was less than 33%.

Melphalan did not appear to have any antitumor effect in the 12 patients who received the drug initially or in the nine patients who received the drug subsequently. Hydroxyurea failed initially in 11 patients and subsequently in 13 patients. It seems unlikely that extending the study to include more patients would demonstrate that either melphalan or hydroxyurea,

TABLE 1.—Objective remissions after chemotherapy in patients with disseminated malignant melanoma

	Chemotherapy		
	No. remissions/No. patients treated		
Treatment	NSC-45388	Melphalan	Hydroxyurea
Primary	2/12	0/12	0/11
Subsequent	0/11	0/9	0/13
Total	2/23*	0/21	0/24

*Does not include 2 patients with responses of less than 33%.

at the dose regimens used in this study, would be of any value in the treatment of patients with disseminated malignant melanoma.

The toxicity which occurred with these three drugs was significant but generally tolerable (table 2). NSC-45388 caused nausea and vomiting in all patients who received it and produced significant but tolerable hematologic depression as previously reported (5,6). Two deaths were associated with the use of NSC-45388; both of these deaths occurred within 3 weeks of treatment and both were accompanied by hematologic depression, particularly platelet depression. One patient had a large melanoma-like lesion adjacent to the carotid artery that was associated with acute and terminal bleeding. The other patient had profound bleeding in the nasopharyngeal region that contributed to his death. One patient who had received NSC-45388 had appreciable alopecia and two of the 23 patients who received this drug complained of facial flushing.

Melphalan was associated with nausea and vomiting in 15 of the 21 patients but, as with NSC-45388, these reactions were self-limited and did not necessitate the use of iv infusions of fluid. Hematologic depression was seen with melphalan, although the depression was less extensive than with hydroxyurea.

Hydroxyurea given by the 5-hour infusion technique caused nausea and vomiting in 16 of the 24 patients who received the drug. The incidence of hematologic depression was higher with this drug than with melphalan or NSC-45388 (table 2). The nadir leukocyte count with hydroxyurea occurred 5.6 days (average) after treatment and generally was temporary.

Of the 35 patients who participated in the study, 27 have died and eight are alive. The median survival time (from the time of treatment) of the patients who died was 4 months (mean, 4.3 months).

TABLE 2.—Toxic effects of chemotherapy in patients with disseminated malignant melanoma

	Chemotherapy		
	Incidence of toxic effect/No. patients treated		
Toxic effect	NSC-45388	Melphalan	Hydroxyurea
Leukocyte depression (<3000 cells/mm³)	7/23	6/21	18/24
Platelet depression (<100,000 cells/mm³)	5/12	3/21	6/24
Gastrointestinal (nausea and vomiting)	23/23	15/21	16/24
Alopecia	1/23	0/21	0/24

DISCUSSION

Disseminated malignant melanoma is one of the most refractory neoplasms; however, occasional responses to treatment reported in the past hardly seem to justify the use of many of the currently available chemotherapeutic compounds in the treatment of this disease. Of the three drugs under study in our randomized clinical trial, only NSC-45388 manifested measurable antitumor effect and, as such, is an agent deserving further clinical trial, perhaps in combination with other chemotherapeutic efforts.

There seems to be little justification for the use of hydroxyurea and melphalan in the dose regimens employed in our study in the management of disseminated malignant melanoma.

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Combination Therapy with Mitomycin C (NSC-26980), 5-Fluorouracil (NSC-19893), and Cytosine Arabinoside (NSC-63878) for Advanced Cancer in Man [1,2,3]

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SUMMARY

The combination of mitomycin C, 5-fluorouracil, and cytosine arabinoside (MFC), which had a marked synergistic effect in the treatment of L1210 mouse leukemia, was used clinically against advanced cancer. Response was assessed according to criteria proposed by Karnofsky. Of 90 patients with various kinds of solid tumors, 17 (19%) had I-B responses and 20 (22%) had I-A responses. Either I-A or I-B responses were observed in 15 (55%) of 27 patients with cancer of the stomach and in nine (60%) of 15 patients with cancer of the colon, indicating that the MFC combination is especially effective against gastrointestinal adenocarcinoma. Bone marrow suppression and gastrointestinal toxicity were observed; however, if the MFC therapy is carried out carefully with adequate intervals between treatments and periodic blood tests, it can be expected to be a safe and useful treatment that is superior to other chemotherapy especially for gastrointestinal cancers.
[Cancer Chemother Rep 56:373–385, 1972]

Although the development of new antitumor agents is essential for the progress of chemotherapy for malignant tumors, studies on the method of administration of available chemotherapeutic agents are also necessary. Recently, chemotherapy with combinations of two or more drugs has been used more extensively (1–7). We have studied combination therapy for malignant tumors both experimentally and clinically (8,9), and our co-workers, Hoshino et al, have reported that a combination of mitomycin C, 5-fluorouracil (5-FU), and cytosine arabinoside (MFC) had a synergistic effect in the prolongation of the lifespan of mice with L1210 leukemia (10). Since this effect was so remarkable, the combination was used for the treatment of advanced cancer in man (11–13).

[1] Received Oct 5, 1970; revised Dec 28, 1971; accepted Jan 27, 1972.

[2] Presented in part at the 17th Annual Meeting of the Japanese Chemotherapy Society in 1969, the 6th International Congress of Chemotherapy in 1969, the 10th International Cancer Congress in 1970, and the 8th Annual Meeting of the Japanese Society of Cancer Therapy.

[3] Cytosine arabinoside: cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride. Drugs used in this study were supplied by Nippon Shinyaku Co., Ltd., and Kyowa Hakko Kogoyo Co., Ltd.

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METHODS AND MATERIALS

A mixture of 0.04–0.08 mg/kg of mitomycin C, 5–10 mg/kg of 5-FU, and 0.4–0.8 mg/kg of cytosine arabinoside, dissolved in physiologic saline or 5% glucose, was given intravenously (iv) once or twice a week. Later in the study, a full dose of each drug was given twice a week for the first 2 weeks and thereafter once a week; leukocyte (wbc) and platelet counts were checked periodically during treatment. When decreases in the counts were observed, the frequency of injections was decreased from once a week to once every other week, and when the observed decreases were severe, the injections were stopped temporarily. Treatment was begun again after recovery of the wbc and platelet counts to normal levels.

Ninety patients with cancers not amenable to surgical treatment received treatment with MFC. Of the 90 patients, 27 had cancer of the lung, 27 had cancer of the stomach, and 15 had cancer of the colon and rectum. The effect of treatment with MFC was judged according to response criteria proposed by Karnofsky (14).

RESULTS

Therapeutic Response

The types of solid tumors that were treated and the observed responses are summarized in table 1. Of 27 patients with lung cancer, four (15%) had I–A responses, and one (4%) had a I–B response. Histologic examination revealed that in all four cases of squamous cell carcinoma of the lung, response to treatment was of the 0–0 (three patients) or 0–A (one patient) category; in five (36%) of 14 cases of adenocarcinoma, response was of the I–B (one patient) or I–A (four patients) category; and in two of eight cases of undifferentiated carcinoma, response was of the I–A category. Of 27 patients with cancer of the stomach, nine (33%) had I–A responses and six (22%) had I–B responses. Of 15 patients with cancer of the colon and rectum, five (33%) had I–A and four (27%) had I–B responses. Two responses (I–A and I–B) were observed in three patients with cancer of the pharynx. One response (I–B) was noted in two patients with cancer of the cervix. Two responses (I–B) were noted in two

TABLE 1.—Response to treatment with MFC of patients with various solid tumors

Diagnosis	No. of patients treated	No. and percent of patients in response category*— 0–0	0–A	0–B	0–C	I–A	I–B
Cancer of the lung	27	16 (59%)	3 (11%)	1 (4%)	2 (7%)	4 (15%)	1 (4%)
						5 (19%)	
Cancer of the stomach	27	8 (30%)	2 (7%)	1 (4%)	1 (4%)	9 (33%)	6 (22%)
						15 (55%)	
Cancer of the colon and rectum	15	4 (27%)	0	0	2 (13%)	5 (33%)	4 (27%)
						9 (60%)	
Cancer of the pharynx	3	1	0	0	0	1	1
Grawitz's tumor	3	3	0	0	0	0	0
Reticulum cell sarcoma	3	3	0	0	0	0	0
Cancer of the cervix	2	1	0	0	0	0	1
Embryonal carcinoma	2	0	0	0	0	0	2
Other cancers	8	4	1	0	0	1	2
Total	90	40 (44%)	6 (7%)	2 (2%)	5 (6%)	20 (22%)	17 (19%)

*Karnofsky (14).

TABLE 2.—Duration of response to MFC therapy

Months	No. of patients in response category— I–B	I–A	Total No. of patients responding (% of patients responding)	
1–2	4	12	16 (43)	25 (67)
2–3	4	5	9 (24)	
3–4	4	1	5 (13)	8 (21)
4–5	1	1	2 (5)	
5–6	1	—	1 (3)	
6–7	1	—	1 (3)	
7–8	—	1	1 (3)	
8–9	—	—	—	
9–12	—	—	—	
12–18	1	—	1 (3)	
18–24	1	—	1 (3)	
Total	17	20	37 (100)	

patients with embryonal carcinoma. In the group of patients with other cancers, one patient with squamous cell carcinoma of the scapula (primary site unknown) and one with retroperitoneal undifferentiated sarcoma had I–B responses. In total, of the 90 patients treated, 40 (44%) had 0–0 responses, six (7%) had 0–A responses, two (2%) had 0–B responses, five (6%) had 0–C responses, 20 (22%) had I–A responses, and 17 (19%) had I–B responses.

The duration of response to MFC therapy, that is, the period from the beginning of a response to the time of recurrence, is shown in table 2. Twenty-five (67%) of 37 patients who responded to MFC treatment (I–A or I–B responses) had responses lasting 1–3 months. Responses of 3–6 months' duration were noted in eight patients (21%), and included more I–B responses than I–A responses. There were very few responses lasting longer than 6 months.

Toxicity

Toxic effects observed during treatment are shown in table 3. Hematologic effects were fairly strong: leukocytopenia with counts of less than 2000 cells/mm^3 was observed in 33% of the patients and counts of 2000–4000 cells/mm^3 were observed in 33% of the patients. Leukocytopenia with counts of less than 2000 cells/mm^3 occurred in 59% of the patients who had I–B responses but in only 23% of the patients who had 0–0, 0–A, 0–B, or 0–C responses. Thrombocytopenia with counts of less than 30,000 cells/mm^3 was observed in 27% of the patients and platelet counts of 30,000–100,000 cells/mm^3 were observed in 31% of the patients. No correlation between response and thrombocytopenia was observed.

Anorexia was observed in 46% of the patients treated, nausea in 34%, diarrhea in 21%, vomiting in 19%, abdominal pain in 9%, epilation in 3%, and severe pigmentation in 3%. Fever, bleeding, and fatigability were observed in a few patients. One case of parkinsonism was observed. Nausea, vomiting, diarrhea, and abdominal pain were observed in a higher percentage of the patients who had I–B responses than in patients who had 0–0, 0–A, 0–B, or 0–C responses. On the other hand, none of these non-hematologic side effects were observed in 33% of the total number of patients treated. There did not appear to be any correlation between type of response and absence of non-hematologic side effects.

Levels of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT),

TABLE 3.—Toxic effects of MFC therapy

Toxic effects	Categories of response: No. of patients:	No. of patients in whom toxic effects occurred* I–B 17	I–A 20	0–0, 0–A, 0–B, 0–C 53	Total 90
Hematologic					
Leukopenia (wbc/mm³)					
<2000		10 (59)	8 (40)	12 (23)	30 (33)
2000–4000		4 (23)	5 (25)	21 (39)	30 (33)
>4000		3 (18)	7 (35)	20 (38)	30 (33)
Thrombocytopenia (platelets/mm³)					
<30,000		5 (29)	2 (10)	17 (32)	24 (27)
30,000–100,000		4 (24)	9 (45)	15 (28)	28 (31)
>100,000		8 (47)	9 (45)	21 (40)	38 (42)
Non-hematologic					
None		5 (29)	7 (35)	18 (34)	30 (33)
Anorexia		7 (29)	9 (45)	25 (47)	41 (46)
Nausea		9 (53)	5 (25)	17 (32)	31 (34)
Vomiting		6 (35)	2 (10)	9 (17)	17 (19)
Diarrhea		5 (29)	5 (25)	9 (17)	19 (21)
Abdominal pain		4 (24)	1 (5)	3 (5)	8 (9)
Epilation		2 (12)	1 (5)	0	3 (3)
Pigmentation		1 (6)	0	2 (4)	3 (3)
Fever		1 (6)	0	1 (2)	2 (2)
Fatigability		0	0	1 (2)	1 (1)
Parkinsonism		1 (6)	0	0	1 (1)
Bleeding		1 (6)	0	1 (2)	2 (2)

*Numbers in parentheses indicate percent of the total number of patients in each response category.

serum glutamic pyruvic transaminase (SGPT), and serum protein were determined before and after therapy. Significant differences caused by MFC therapy alone were not evident.

Case Reports

Patient No. 1—A 42-year-old woman noted swelling in her left cervical lymph node in September 1968. Since August of that year, she had lost almost 10 kg of body weight. A chest roentgenogram taken in October showed many metastatic tumor shadows. A diagnosis of carcinoma of the uterine cervix with metastases to the lungs and cervical lymph node was made (*see* fig 1). She had fever with a body temperature higher than 38 C every day after hospitalization. A thumb-sized tumor was palpated on the left side of her neck; the largest tumor shadow in the lung was 7.0 × 7.0 cm in diameter.

From November 22 to December 6, 30-mg doses of bleomycin (NSC-125066) were administered twice a week to a total dose of 150 mg. During the therapy, the tumors increased in size, however, and the largest one increased to 7.8 × 7.1 cm in diameter (fig 1–A). The tumor of the cervix increased in size from 8 × 8 cm to 10 × 10 cm. MFC therapy was begun on December 12. After three doses of MFC had been given, the main tumor in the lung decreased in size to 6.0 × 6.0 cm, and the tumor in the left wall of the pelvis decreased in size to 8 × 8 cm, although nausea and vomiting continued. Marked degeneration of cancer cells was observed in the biopsy from the vaginal part of the uterus (cervix uteri). After the third treatment, MFC therapy was continued with longer

intervals between courses because of the occurrence of toxic effects. As an unusual side effect, symptoms of Parkinson's disease were observed, ie, irritation, tremor, slowness of movement or speech, and salivation. These side effects disappeared when trihexyphenidyl HCl (Artane), antihistamines, and atropine sulfate were administered with the MFC. After the sixth administration of MFC, the swelling of the cervical lymph node disappeared, the main tumor in the lung decreased in size to 3.5 × 3.5 cm and no other tumors were visible on the roentgenogram. The tumor in the left wall of the pelvis decreased in size to 6 × 6 cm and the uterus became movable. The tumor of the cervix disappeared and no cancer cells were observed in the biopsy specimen. After the tenth dose of MFC had been given, the main tumor in the lung decreased in size to 3 × 3 cm, the uterus became normal in size, and the pelvic tumor also decreased in size to 5 × 5 cm. On March 3, the leukocyte count decreased to 400 cells/mm^3 and MFC therapy was interrupted after the 13th treatment (a total of 52 mg of mitomycin C, 6500 mg of 5-FU, and 520 mg of cytosine arabinoside had been given). At that time, the size of the main tumor was 2.0 × 2.0 cm (fig I–B). The patient's general condition improved, and she was discharged from the hospital.

On April 17, it was noticed that the main lung tumor had increased in size to 3.5 × 3.0 cm and the pelvic tumor was also enlarged. MFC therapy was begun again on April 24; five doses were given. Subsequently, radiation therapy to the lung, pelvis, and neck was also carried out; however, the patient's general condition and hematologic status became worse and she died on October 21 without a second remission.

Patient No. 2—A 73-year-old man had had anorexia since the beginning of 1969; on October 4, 1969, a roentgenogram indicated that there was a large tumor at the pylorus. Coronary insufficiency was also recognized. Since the patient refused surgical treatment, MFC therapy was begun on March 16, 1970. After the fifth administration of MFC, a roentgenogram revealed that the tumor in the patient's stomach had decreased in size from 9.2 × 7.0 to 6.0 × 5.0 cm; however, the leukocyte count had also decreased (to 1400 cells/mm^3) and the therapy was interrupted temporarily. Treatment was resumed after recovery of the wbc to normal and was continued intermittently (one dose every 2 or 3 weeks). After the ninth administration of MFC, the tumor shadow decreased further in size (fig 2). The patient's general condition was satisfactory and he was discharged from the hospital. During the intermittent administration of MFC, no severe decrease in the wbc was observed for 1 year. The tumor began to increase in size 15 months after the start of MFC therapy.

Patient No. 3—A 54-year-old woman noted soft and bloody stools and came to the hospital on March 7, 1970. Roentgenographic studies of the colon revealed a tumor 6.0 × 5.5 cm in size on the sigmoid colon (fig 3). Surgical resection of the tumor was attempted on July 6, but it was not successful because the fist-sized tumor had infiltrated and become fixed in the retroperitoneum, and multiple metastases were observed in the lymph nodes around the aorta and in the liver; a colostomy was performed instead. Although 100 mg/day of cyclophosphamide [6] was given to a total dose of 2300 mg, the tumor increased in size to approximately 25 × 25 cm and the girth of the patient's abdomen was 88.5 cm. MFC therapy was started on November 18. After the fourth treatment, the tumor began to decrease in size. After the sixth treatment, the wbc decreased to 1200 cells/mm^3 and the thrombocyte count decreased to 33,000 cells/mm^3; vomiting continued and the patient's general condition became worse. The counts returned to normal 2 weeks after the sixth treatment, and the patient's condition improved in general. At the same time, there was a marked decrease in the size of the abdominal tumor. MFC therapy was then started again and continued once a week intermittently. No decreases in wbc or thrombocyte count were observed with this course of treatment. The tumor could not be palpated on February 5 after the tenth administration of MFC. The girth of the patient's abdomen had decreased to 76 cm and her general condition improved greatly. Roent-

[6] Cyclophosphamide: NSC–26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan; endoxan.

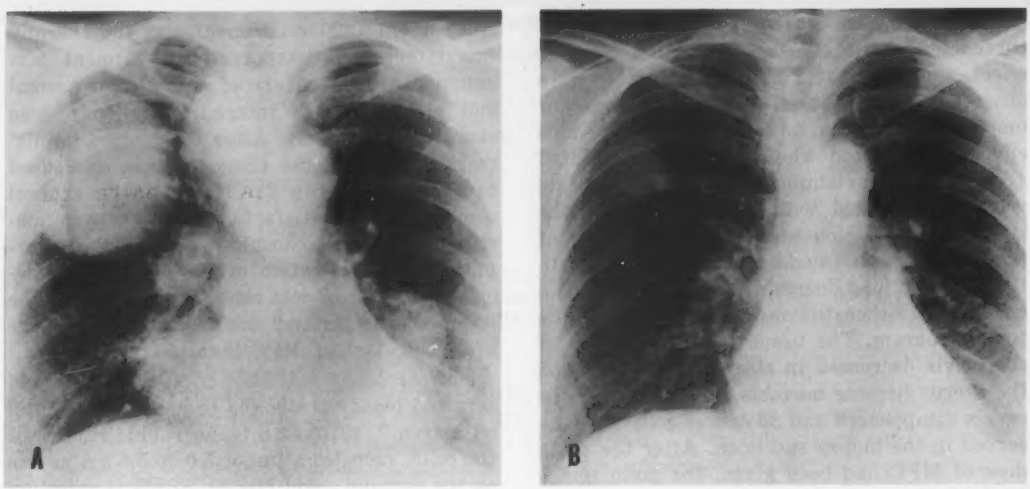

FIGURE 1.—A. Patient No. 1 before MFC therapy (December 7, 1968). B. Same patient after MFC therapy (February 2, 1969).

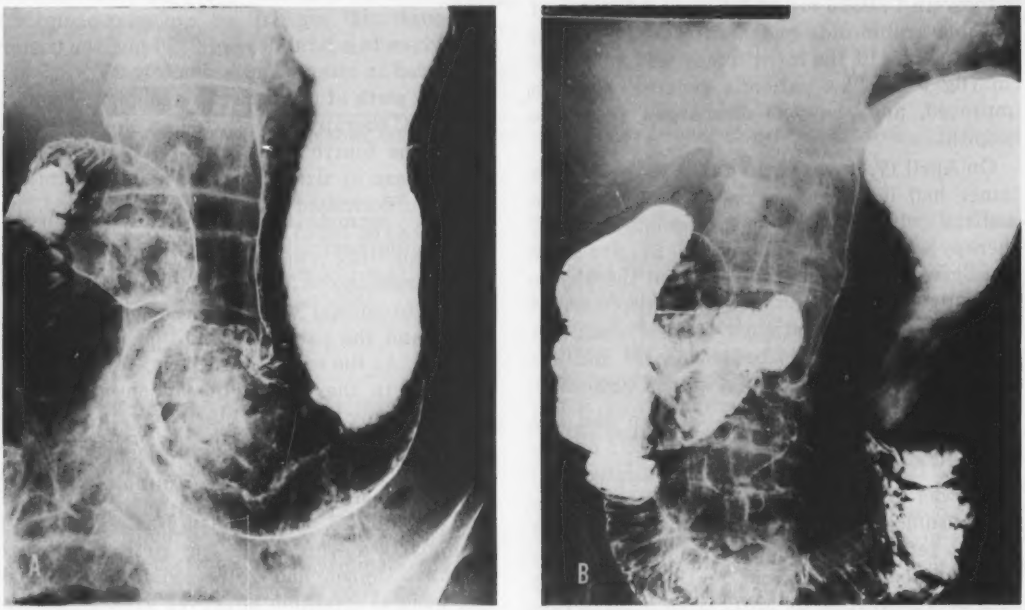

FIGURE 2.—A. Patient No. 2 before MFC therapy (February 18, 1970). B. Same patient after MFC therapy (July 11, 1970).

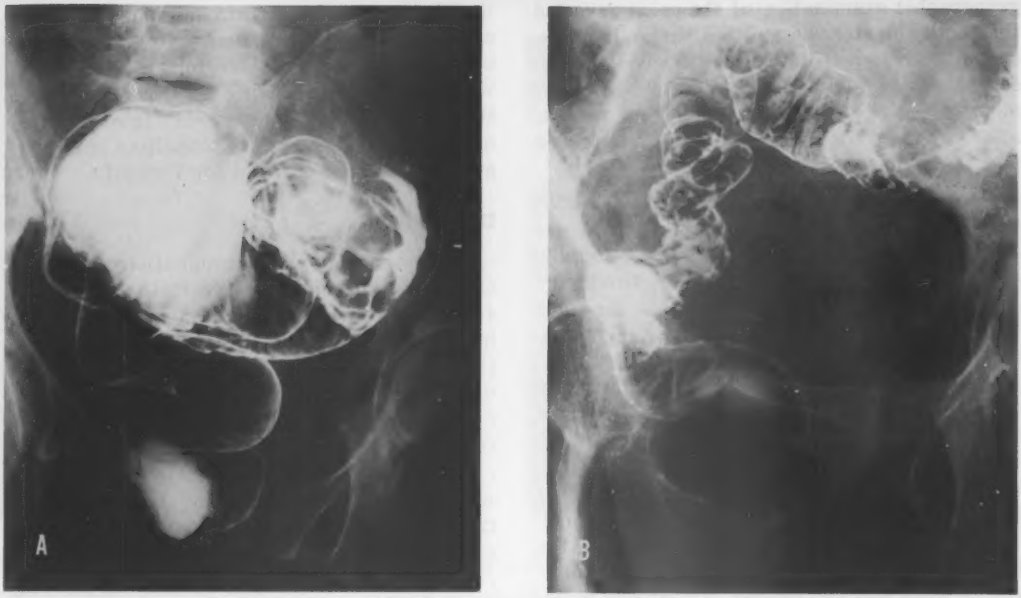

FIGURE 3.—A. Patient No. 3. Barium enema before surgical treatment (May 13, 1970). B. Barium enema after MFC therapy (February 25, 1971).

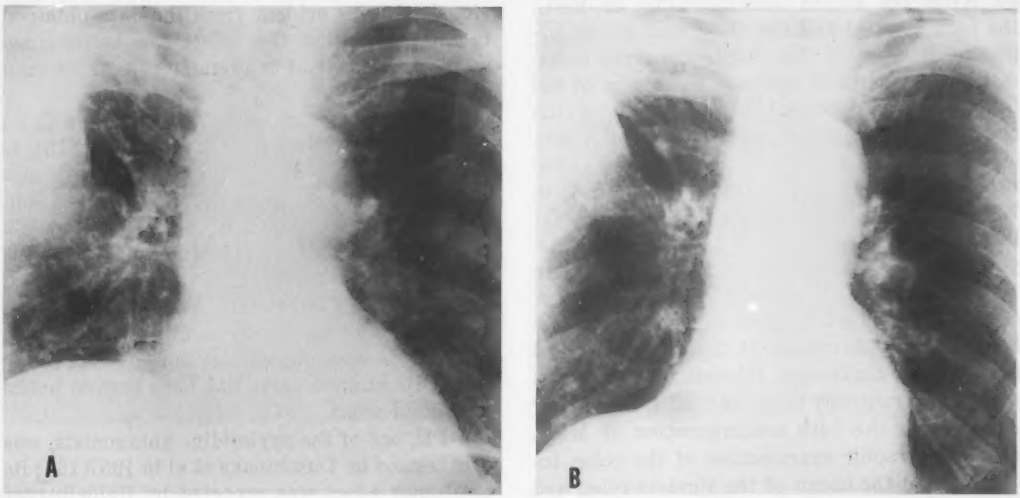

FIGURE 4.—A. Patient No. 4 before MFC therapy (September 29, 1970). B. Same patient after MFC therapy (December 19, 1970).

genographic examination of the colon revealed that both the stenosis and the tumor had disappeared. No gastrointestinal toxicity was observed during maintenance therapy; however, albuminuria and edema occurred. Four months after the tumor had disappeared, it reappeared and began to increase in size.

Patient No. 4—A 47-year-old man had felt pain at the right side of his chest wall since May 1970, and the pain had increased gradually. In June, he left his job because he tired very easily. In August, suppuration from the anus was noticed and a diagnosis of periproctal abscess was made. A chest roentgenogram revealed a tumor shadow on the right side of the upper chest field, and osteolytic change at the second and third ribs (fig 4). An 8 × 6-cm swelling on the right side of the upper chest wall caused the patient great pain. Biopsy of the chest wall tumor revealed adenocarcinoma. The liver was palpable 4 fingerbreadths in the epigastrium and the surface was irregular. Ascitic fluid was also noticed. Bloody and mucoserous stool was observed. Roentgenographic examination of the colon revealed a defect of the sigmoid colon (fig 5). A diagnosis of carcinoma of the colon with metastases to liver and lung (involving the chest wall) was made. MFC therapy was begun on September 30. After the fourth administration of MFC, the pain stopped and the chest wall tumor began to decrease in size. After the sixth treatment, the amount of ascites and the size of the liver decreased noticeably, the tumor of the chest wall disappeared, and the patient's general condition greatly improved. The total serum protein level increased from 5.4 to 7.6 g/100 ml, the albumin-globulin ratio (A/G) increased also from 0.4 to 0.8, and the alkaline phosphatase level decreased from more than 100 to 9.7 King-Armstrong units/100 ml. A chest roentgenogram revealed that there had been marked shrinkage of the tumor shadow and that the diaphragm (elevated before treatment) had returned to its normal position (fig 4–B). After the 14th administration of MFC, roentgenographic examination of the colon indicated that the tumor of the sigmoid colon had completely disappeared (fig 5–B). A large defect of the right lobe of the liver, observed in the liver scan before treatment, had greatly decreased in size (fig 6). Leukocytopenia, thrombocytopenia, and non-hematologic side effects were not observed during MFC therapy. MFC treatment was administered intermittently once every 2 weeks at the outpatient clinic and good remission continued for 7 months.

DISCUSSION

Chemotherapy with combinations of drugs should be expected to have certain advantages: (*a*) the use of a combination of drugs (each having a different mechanism of antitumor activity) can be expected to produce a spectrum of antitumor action wider than that produced by each drug alone; (*b*) the variation in susceptibility of the different phases of the mitotic cycle of malignant cells should be overcome with the use of a multiple of drugs (each attacking a different phase); (*c*) certain combinations can be expected to show synergistic effect; and (*d*) the use of a combination of drugs (each having a different toxic effect) may reduce the total toxicity and enhance the antitumor effect.

In mice with L1210 leukemia, the advantage of treatment with MFC was the synergistic effect of the combination on the prolongation of lifespan, corresponding to (*c*) described previously. It was evident from the data obtained in this study that this effect can be obtained in the treatment of malignant tumors in man as well.

The mechanism of action of mitomycin C, an antibiotic isolated by Hata et al in 1956 (15), is strong inhibition of DNA synthesis (16). According to a recent observation (17), it is considered that enzymatically reduced mitomycin C reacts with DNA and that the activity of the DNA polymerase is inhibited by the cross-linked DNA, resulting in inhibition of DNA synthesis. This agent has been investigated and has been in wide clinical use in Japan for many years. Its clinical value has been proved by its antitumor effect.

5-FU, one of the pyrimidine antagonists, was synthesized by Duschinsky et al in 1957(18); its antitumor effect was reported by Heidelberger et al (19). It is thought that 5-FU is converted to the deoxyribonucleotide (FUDRP) which

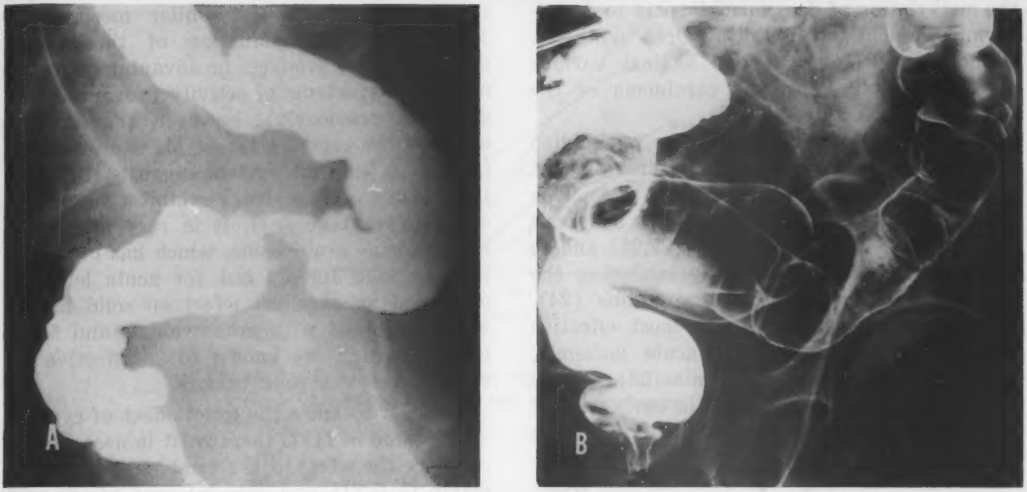

FIGURE 5.—A. Patient No. 4. Barium enema before MFC therapy (September 9, 1970). B. Barium enema after MFC therapy (January 12, 1971).

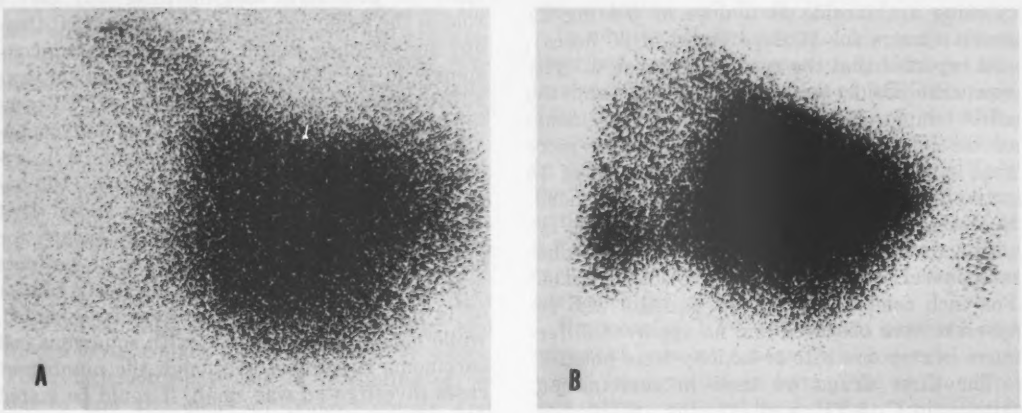

FIGURE 6.—A. Patient No. 4. Liver scan before MFC therapy (September 16, 1970). B. Liver scan after MFC therapy (February 2, 1971).

blocks thymidylate synthetase, thus preventing the methylation of deoxyuridylic acid to deoxythymidylic acid and inhibiting DNA synthesis. Clinically, 5-FU is effective against various kinds of cancer, especially carcinoma of the colon, ovary, and breast (20).

Cytosine arabinoside, one of the pyrimidine analogs, was first shown to be active against animal tumors by Evans et al in 1961 (21). This compound is thought to inhibit DNA synthesis by inhibiting DNA polymerase (22,23) and to prevent formation of DNA by inhibiting the conversion of cytidine to deoxycytidine (24). This compound is one of the most effective drugs for the treatment of acute leukemia, especially acute myeloid leukemia (25). On the other hand, it was reported in the early days of investigation of this compound that it had no significant effect on solid tumors (26–29). Thereafter, the drug was used for the treatment of acute leukemia only. In 1969, Talley et al reported that seven (16%) of 44 patients with various kinds of cancer had objective responses to treatment with the drug (no complete remissions were obtained) (30). The authors of that paper noted that three of four patients with carcinoma of the stomach whose cases could be evaluated did respond to treatment. Recently, Burke et al (31) treated patients with various kinds of solid tumors with cytosine arabinoside at a dose of 0.4 mg/kg every 8 hours for 10 days (total of 30 doses), and reported that the rate of response to cytosine arabinoside was less than 10% and the effect temporary; of 37 patients with carcinoma of the colon, only three responded with more than a 50% decrease in tumor size, and of 14 patients with breast cancer, only one responded. Recently, Stolinsky et al (32) tried to use cytosine arabinoside combined with cyclophosphamide, vincristine, or trimethylcolchicinic acid.[7] For each combination, a few patients who responded were observed and no apparent differences in response rate or toxicity were noted.

The three drugs we used in combination, mitomycin C, 5-FU, and cytosine arabinoside, resemble each other in that all attack DNA synthesis by relatively similar mechanisms. Therefore, the combination of these three drugs probably produces no advantage in terms of a wider spectrum of activity (advantage [*a*] described previously); however, the excellent effect of the combination of MFC observed in the L1210 leukemia system suggests the possibility that the three drugs exhibit a synergistic effect (advantage [*c*]). It is very interesting that cytosine arabinoside, which has been used not for solid tumors but for acute leukemia only, had an excellent effect on solid tumors when combined with mitomycin C and 5-FU, both of which are known to be effective for the treatment of solid tumors.

In order to know the exact effect of cytosine arabinoside in MFC therapy, it is necessary to compare the effect of the combination of mitomycin C, 5-FU, and cytosine arabinoside with the effect of the combination of mitomycin C and 5-FU (MF). We have performed this comparative study and our results have indicated that MFC therapy is more potent than MF therapy for gastrointestinal cancer (33). The data reported by Talley et al (28), ie, that patients with cancer of the stomach responded to treatment with cytosine arabinoside (although the number of cases reported was small), suggests the possibility that the drug may play a role in the enhanced effectiveness of MFC therapy for stomach cancer. The percentage of response to MFC therapy for lung cancer was 33%; the percentage of response to METT combination therapy (mitomycin C, cyclophosphamide, thioTEPA, and chromomycin A_3), reported by us previously (9), was 40% (100 patients).[8] Therefore, it cannot be stated that MFC therapy is superior to METT therapy for lung cancer; however, in a group of patients with lung cancer treated with MFC, six (43%) of 14 patients with adenocarcinoma responded, while none of four patients with squamous cell carcinoma responded. Although the number of cases investigated was small, it could be stated from these data that, in addition to the fact that MFC therapy is very effective for gastro-

[7] Vincristine: NSC–67574, leurocristine, sulfate (1:1).

Trimethylcolchicinic acid: NSC–36354; trimethylcolchicinic acid, methyl ether, L-tartrate (1:1), hydrate.

[8] ThioTEPA: NSC–6396; phosphine sulfide, tris(1-aziridinyl)-; triethylenethiophosphoramide.

Chromomycin A_3: NSC–58514; Toyomycin.

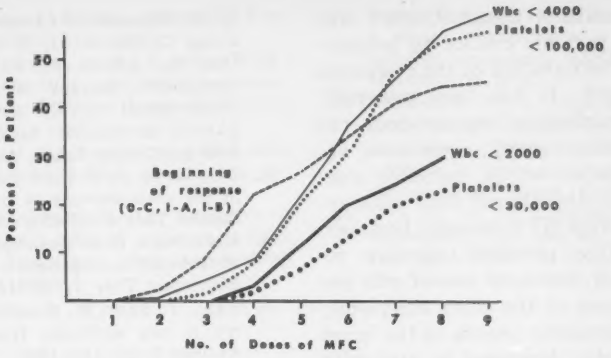

FIGURE 7.—Relationship between the development of response (0–C, I–A, I–B), leukocytopenia, and thrombocytopenia during MFC therapy.

intestinal cancer, it may also be effective in general against adenocarcinoma occurring in various organs.

The most serious toxic effect of MFC treatment is bone marrow suppression. The most severe toxic effect of treatment with mitomycin C (or cytosine arabinoside) is bone marrow suppression also. The main toxic effects of 5-FU are stomatitis, diarrhea, and bone marrow suppression. Therefore, advantage (*d*) of combination therapy mentioned previously cannot be expected in the MFC therapy because the side effects of each of the three drugs are similar in that all three cause bone marrow suppression. In almost all patients treated with MFC for long periods, leukocytopenia and thrombocytopenia were observed frequently. These hematologic toxic effects were especially severe with the treatment schedule used in an early stage of this investigation (ie, full doses of MFC twice a week). Subsequently, half the full dose was administered twice a week or the full dose was administered once a week; this resulted in only slight decreases in wbc and thrombocyte counts but less antitumor effect. The relationship between the beginning of response and the onset of leukocytopenia and thrombocytopenia is shown in figure 7. In 22% of the patients who had I–B, I–A, or 0–C responses, response occurred after the fourth administration of MFC. At that total dose, leukocytopenia with counts of less than 4000 cells/mm^3 occurred in 8% of the patients and leukocytopenia with counts of less than 2000 cells/mm^3 occurred in 3%; however, after the fifth treatment, severe leukocytopenia with counts of less than 2000 cells/mm^3 was seen in 12%. Response was obtained in 27% of the patients. Therefore, in the latter part of the study, the MFC treatment schedule was improved as follows: for the first 2 weeks, the full dose was given twice a week; subsequently, it was given once a week and hematologic status was evaluated periodically.

In addition to the hematologic toxic effects described previously, gastrointestinal toxicity is also a serious side effect of MFC therapy. This toxicity occurs with each of the three drugs, but especially with 5-FU, which is known to damage the intestine. The fact that 21% of the patients treated with MFC had diarrhea could be explained mostly by the action of 5-FU. Anorexia, nausea, or vomiting usually occurred on the day of injection and sometimes continued the next day.

Response to MFC therapy in patients with advanced cancers against which single drugs would be expected to have little or no effect was often remarkable; however, in an early stage of the investigation, the duration of response was rather short because the therapy had to be interrupted due to the severe leukocytopenia or thrombocytopenia which occurred. In contrast to this, in the latter part of this study,

a long-term, intermittent administration was carried out (with periodic checks on hematologic status), and the duration of the responses became much longer. It has been generally known that gastrointestinal cancer does not respond well to chemotherapy; even when abdominal tumors decreased in palpable size, roentgenographic studies did not show substantial improvement. With MFC therapy, however, roentgenograms often revealed objective response. The primary lesion of one of our patients with carcinoma of the colon completely disappeared and metastatic lesions in the lungs and liver also greatly decreased in size with MFC therapy.

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Clinical Trials With 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196) Given Intravenously [1,2]

Charles M. Bagley, Jr.,[3] George P. Canellos,[3] Robert C. Young,[3] Joseph F. Gallelli,[4] and Vincent T. DeVita, Jr.[3]

SUMMARY

Clinical trials with 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196) given intravenously to 32 patients with various tumors suggest that the maximum tolerated dose is 5 g/m² divided into five consecutive daily injections and repeated at 5–6-week intervals. The principal dose-limiting toxic effect was severe nausea and vomiting. Pain and flushing after administration were often severe. The time of onset and the degree of severity of delayed bone marrow toxicity (nadir, 31–35 days) were erratic, but the toxicity was reversible and usually tolerable. One patient with lymphosarcoma had an objective response. Phase II trials with NSC-82196 in patients with lymphoma and melanoma appear warranted.
[Cancer Chemother Rep 56:387–391, 1972]

5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (NSC-82196), one of a series of imidazole derivatives synthesized by Shealy et al (1,2), has shown activity against the mouse neoplasms L1210 leukemia, AKR lymphoma, Sarcoma 180, Adenocarcinoma 755, and rat Walker carcinoma 256 (1,3,4). A related drug, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388), which also showed activity against L1210 leukemia, has produced approximately 20% responses in patients with metastatic melanoma, using either a single intravenous (iv) dose or iv doses given every 5 days (5,6). Comparative studies in L1210 leukemia show that NSC-82196 is much more effective than NSC-45388 in reducing the number of viable tumor cells and prolonging survival time (7,8). Moreover, L1210 variants resistant to NSC-45388 retain full sensitivity to NSC-82196, whereas variants resistant to NSC-82196 are highly resistant to NSC-45388 (8).

The principal toxic effects observed in animal studies included reversible myelosuppression, emesis, proteinuria, elevated liver function tests, tachycardia, and hypotension.[5]

A clinical trial of NSC-82196 given orally resulted in brief objective responses in three patients who had lymphoma and no responses in 26 other patients who had various tumors (9). The only toxic effects observed were nausea and vomiting in a majority of the patients and mild bone marrow depression in a minority of the patients when the highest dose level of 1

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[2] NSC-82196 was supplied by Drug Research and Development, Chemotherapy, National Cancer Institute (NCI).

[3] Solid Tumor Service, Medicine Branch, Chemotherapy, NCI, Bethesda, Md.

[4] Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, Md.

[5] Contractor's report to Drug Research and Development (formerly Cancer Chemotherapy National Service Center), Chemotherapy, NCI, from South Shore Analytical and Research Laboratory, Oct 27, 1969.

g/m²/wk was used (9). Clinical pharmacologic studies indicated that the oral form was erratically and poorly absorbed, and attention was directed to formulating an iv preparation. This report deals with the initial clinical trials using the iv form of NSC-82196.

METHODS

Thirty-three drug trials were performed in 32 patients who had widely metastatic cancer. All established therapeutic modalities had been exhausted for these patients. Their diagnoses were: melanoma, 15 patients; lymphosarcoma, four; carcinoma of the colon, four; osteogenic sarcoma, three; and one each with carcinoma of the breast, carcinoma of the kidney, carcinoma of unknown primary, hepatoma, and chronic lymphocytic leukemia. All patients had received extensive chemotherapy 1 or more months earlier, except for eight patients with melanoma, two with osteogenic sarcoma, and one with carcinoma of the kidney, all of whom received NSC-82196 as their primary treatment.

NSC-82196 in 100- or 300-mg vials was reconstituted with 0.01 M hydrochloric acid to a final concentration of 20 mg of drug per milliliter, and was administered at once by rapid push into a running iv infusion. Early in the trials, a single dose of NSC-82196 was administered, and if no prohibitive toxicity was observed by 2 weeks later the same dose was given at weekly intervals. Because of the discovery of delayed toxicity later in the study, the schedule was changed to administration of a single course of therapy, followed by a 6-week observation period, and continuation of further NSC-82196 treatment at the same dose level at 6-week intervals. Blood cell counts were obtained weekly or more often if necessary, and tests of hepatic and renal function were repeated at 1- or 2-week intervals and before each drug cycle. Tumor response was assessed by physical examination and roentgenographic studies, if possible. Partial response was defined as a greater than 50% shrinkage of the product of two diameters of all measurable tumors, lasting longer than 1 month. The drug doses given and the number of patients and doses at each level are shown in table 1. At doses up to 1100 mg/m², the entire dose was given in one bolus. Because of severe nausea occurring after single doses greater than 1000 mg/m², higher doses were separated into five equal boluses on consecutive days.

TABLE 1.—Patient distribution and hematologic toxic reactions at each dose level of NSC-82196

Dose* (mg/m²)	No. of patients	No. of doses	Wbc count			Platelet count		
			< 4000 cells	< 2000 cells	Median nadir	< 100,000 cells	< 20,000 cells	Median nadir
Doses given in single bolus								
100–400	11	46	0	—	—	0	—	—
600	3	16	1	1	1500	1	0	70,000
800	1	11	0	—	—	0	—	—
1100	2†	9	0	—	—	1	1	20,000
Doses separated into 5 daily boluses								
1500	3	12	0	—	—	0	—	—
2200	2	5	0	—	—	0	—	—
3000	2†	2	2	1	1500; 3400	2	2	15,000;20,000
4000	2	3	0	—	—	0	—	—
5000	7	13	3	2	3600 (range, 1500–7800)	5	0	36,000 (range, 31,000–165,000)

*Doses $\leq$ 1100 mg/m² given in one bolus. Doses $\geq$ 1500 mg/m² divided into 5 boluses on consecutive days.
†One patient had extreme marrow replacement from his disease (melanoma and marked myelosuppression from treatment with NSC-82196.

RESULTS

Immediate Toxicity

At every dose level rapid administration of NSC-82196 produced local venous pain, which in some patients became severe enough in the higher doses to appreciably delay drug administration. Five patients had objective phlebitis and sclerosis of the veins used for injections, and one patient was suspected of having a small pulmonary embolism on the fourth of 5 days of consecutive drug administration. When single boluses greater than 400 mg/m² were given, almost all patients had moderate or severe nausea and vomiting starting 0–4 hours after administration and lasting up to 48 hours. This was partially relieved by antiemetics. Vomiting was severe enough in two patients to require cessation of therapy. (One of these had bowel metastases with melanoma and had had occasional vomiting before receiving NSC-82196.) A majority of the patients receiving single boluses greater than 600 mg/m² had severe flushing of the face, neck, and thorax lasting up to 1 hour and often accompanied by lightheadedness. Slight or no hypotension was documented during most flushes, but one patient receiving 1000 mg/m² in one bolus had syncope with loss of consciousness. Syncope did not recur after subsequent treatment of this patient with the 5-day schedule. The severity of vomiting (but not of flushing) tended to increase throughout the 5-day drug administration periods.

Delayed Toxicity

The only observed delayed toxic reaction to NSC-82196 was myelosuppression (table 1). The occurrence and severity of leukopenia and thrombocytopenia were unpredictable, and there were striking variations in their time of onset but relatively little variation in the time to recovery.

The median time to the nadir white blood cell (wbc) count was 28 days (range, 8–39), and the median time to recovery was 35 days (range, 34–53). The median time to nadir platelet count was 31 days (range, 8–35), and the median time to recovery was 38 days (range, 36–45). In each individual patient, leukopenia and thrombocytopenia tended to occur in close sequence or simultaneously. In general, patients who had marrow infiltration by tumor or who had received previous intensive chemotherapy tended to have more severe and earlier myelosuppression, but the trend was not statistically significant. The hemoglobin level fell an average of 1.1 g/100 ml in the 2 weeks after the first dose of NSC-82196 and showed no consistent change thereafter. One patient with advanced lymphosarcoma received 5000 mg/m² and had an early fall in wbc count, reaching one nadir 14 days after therapy, recovery to a normal count on Days 16–25, and a second, deeper nadir 31 days after therapy. No other chemotherapy had been given, and although the patient was subsequently seriously ill with pneumonia, the clinical course did not seem to explain the striking fluctuation in wbc counts. This patient's platelet count showed only a single nadir on Day 24. No other patient had a similar course of leukopenia.

Although occasional abnormalities were noted in tests of renal and hepatic function and determinations of proteinuria, in most cases urinary tract infections or hepatic tumor metastases were present. No renal or hepatic abnormalities could be definitely ascribed to treatment with NSC-82196. No mucositis, hair loss, myalgia, or fever was noted.

RESPONSE

One objective response occurred in the patient who had advanced lymphosarcoma refractory to all established therapy including high-dose cyclophosphamide and combination drug therapy. This patient had a marked shrinkage of abdominal and superficial nodes and a loss of ascites after a 5-day course of treatment (total dose, 5000 mg/m²). One other patient with advanced refractory lymphosarcoma who received 5000 mg/m² of NSC-82196, had a slight regression of disease. Of the patients receiving the higher doses of 3000–5000 mg/m² of NSC-82196, no responses were noted among those who had melanoma (6), carcinoma of the kidney (1), osteogenic sarcoma (1), or carcinoma of the colon (1).

DISCUSSION

Physical characteristics of and several assay techniques for NSC-82196 have been described (2,10). NSC-82196 is virtually insoluble in water, but sufficiently soluble in dilute hydrochloric acid to permit formulation for iv use (10). Ultraviolet light catalyzes rapid conversion of NSC-82196 to 2-azahypoxanthine and *nor*-nitrogen mustard, via the intermediate, 5-diazoimidazole-4-carboxamide (11).[6] However, in the dark, in brown bottles, and in vivo, NSC-82196 is more slowly converted by internal cyclization to a "transformation product," NSC-112970 (9,10). Oral absorption of NSC-82196 is incomplete and erratic in man and animals (3,9,11), and no organ localization has been found (11). The plasma half-life of the drug in mice has been estimated to be 12 minutes (12), and most metabolites appear in the urine (9,11).

NSC-82196 enters cells rapidly by passive diffusion (13). The mechanism of cytotoxicity is unknown, but the lack of schedule dependency in the treatment of L1210 leukemia (3) and the first-order kinetics of L1210 cell kill (14) suggest that there is no cell-cycle dependency. In the L1210 system, NSC-82196 is cross resistant with the nitrosoureas (including streptozotocin, NSC-85998) and partially cross resistant with alkylating agents (8),[7] but it is not cross resistant with antimetabolites (8). Equivalent doses of NSC-82196 were much more toxic to AKR lymphoma cells than to normal marrow cells (15). Adjuvant radiotherapy (300 rads, whole body) to mice bearing L1210 leukemia treated with NSC-82196 did not improve survival time over therapy with NSC-82196 alone (Arseneau, J., and Canellos, G. P., personal communication, 1971).

The initial phase I studies of NSC-82196 using the oral preparation were hampered by the apparently erratic absorption of the drug. Using the parenteral formulation, the acute side effects (particularly nausea) proved to be as dose limiting as bone marrow depression. Because of the instability of NSC-82196 in light and its low solubility, rapid administration of large amounts became necessary. These technical obstacles and the immediate toxic effects of boluses greater than 1000 mg/m^2 led to the separation of the total dose into consecutive daily boluses. Using a total dose of 5000 mg/m^2, technical difficulties with the administration of the 1 g/m^2 bolus, severe immediate toxic reactions, and erratic, but occasionally severe myelosuppression again limited further dose escalation.

The immediate and delayed toxic effects of NSC-82196 appear to be very similar to those reported for NSC-45388, although the nadir of leukopenia and thrombocytopenia with NSC-45388 occurred somewhat earlier (5,6). It is also interesting to note that the myelosuppression from NSC-82196 occurs with approximately the same time delay as is observed with BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea; NSC-409962) (17), one of the few drugs showing marked cross resistance with NSC-82196 in animal tumors (8). Although nausea, vomiting, and pain after administration of large doses of NSC-82196 were severe and dose limiting, they were adequately tolerated by most patients when treatment was given at 6-week intervals. A total dose of 5 g/m^2, given as a 5-day course iv, appears to be the maximally tolerated clinical dose.

The response noted in this study in one of two patients with lymphoma treated with adequate doses, and the previously observed response of lymphomas to NSC-82196 given orally (9), suggest that NSC-82196 may be useful in the treatment of this disease. Too few patients with other tumors have been treated with maximally tolerated doses of NSC-82196 to draw conclusions about the drug's usefulness. Because of the current experiences with NSC-45388, further trials appear indicated in patients who have malignant melanoma and other tumors.

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[6] 2-Azahypoxanthine: NSC-22419; 7*H*-imidazo[4,5-*d*]-*v*-triazin-4-ol, monohydrate.

Nor-nitrogen mustard: NSC-10873; diethylamine, 2, *v*-triazin-4-ol, monohydrate.

5-Diazoimidazole-4-carboxamide: NSC-22420.

[7] Contractor's report to the Drug Evaluation Branch, Drug Research and Development (formerly Cancer Chemotherapy National Service Center), Chemotherapy, NCI, from Microbiological Associates, Inc., Sept 1971.

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Initial Clinical Study of Tris(2,2-dimethyl-1-aziridinyl)phosphine Oxide (TEPA-132)[1,2]

Arnold Mittelman, John Plager, and Gerald P. Murphy [3]

SUMMARY

Tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (TEPA-132), an alkylating agent, was chosen for clinical trial because of its superior performance in animal tumor systems. This, 2,2-dimethyl derivative of triethylenephosphoramide was also considered to be less toxic than its parent compound. A safe therapeutic dose, 18 mg/kg given intravenously every 2 weeks, was used. At this dose level, mild leukopenia and thrombocytopenia were observed 1 week after the initial dose was given, with complete recovery at 2 weeks. Major toxic effects noted at the 18 mg/kg dose level and greater were leukopenia and thrombocytopenia. Localized venospasm and peripheral vein thrombosis at the infusion site were common. Nausea and vomiting immediately after drug infusion occurred in all patients receiving the therapeutic dose. No other toxic manifestations were noted. One patient with renal cell carcinoma had an objective remission of her disease.
[Cancer Chemother Rep 56:393–399, 1972]

Munson et al (1) have described the synthesis and autitumor activity of a new alkylating agent, the triethylenephosphoramide analog, TEPA-132 (fig 1).[4] Triethylenephosphoramide and thioTEPA are well-known agents whose carcinolytic effects have been described (2,3). TEPA-132, a 2,2-dimethyl-substituted compound, was developed after observing the enhanced antitumor activity and reduced hematologic toxicity of the methyl analog, meture-

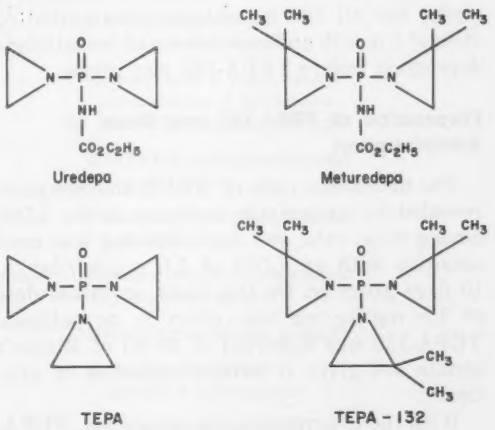

FIGURE 1.—Structural formulas for uredepa, meturedepa, triethylenephosphoramide (TEPA), and TEPA-132. Note the location of the substituted methyl groups.

[1] Received Nov 2, 1971; accepted Mar 1, 1972.

[2] Supported in part by grant RR-262 from General Clinical Research Centers Branch, Division of Research Resources, National Institutes of Health.

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[4] Chemical nomenclature and NSC numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

depa, when compared to the parent compound, uredepa (fig 1) (1). TEPA-132 demonstrated excellent activity against Adenocarcinoma 755, L1210 leukemia, P1534 leukemia, Sarcoma 180, Ehrlich adenocarcinoma, and Dunning ICR 741 tumor (1). The curative action in mice with L1210 leukemia was of particular interest because of th relatively poor activity of the parent co pound, triethylenephosphoramide (1). Mihich and Simpson [5] have confirmed these initial findings in the rodent tumor screens. These workers performed a toxicologic study of TEPA-132 in rodents and dogs, and prompted by these findings, a phase I clinical pharmacologic study of TEPA-132 was carried out.

MATERIALS AND METHODS

Patients

Twenty-four patients were treated with varying dose schedules of TEPA-132 given intravenously (iv). All were hospitalized and their cases initially evaluated in the General Clinical Research Center, Department of Surgery, Roswell Park Memorial Institute. Patients and their diagnoses are listed in table 1. Most patients had received some type of therapy for their metastatic disease before entering this study, and all had a treatment-free period of at least 1 month and no evidence of hematologic depression before TEPA-132 was given.

Preparation of TEPA-132 and Mode of Administration

The toxicologic data of Mihich and Simpson[5] revealed an appreciable variation in the LD50 among mice, rats, and dogs. The dog was most sensitive with an LD50 of 5.0 mg/kg/day × 10 days given iv. On this basis an initial dose of 1.5 mg/kg/wk was given iv to patients. TEPA-132 was dissolved in 30 ml of Ringer's lactate and given iv within 5 minutes of solution.

With the progressive dose escalation, TEPA-132 was diluted with 200–300 ml of Ringer's lactate and given iv in a 20-minute period. The rapidity of the infusion and the pH, of the infused solution were determined by the moderate hydrolysis of TEPA-132 that is known to occur in aqueous solution (if < pH 5). Under these conditions of preparation and administration the compound was biologically active.

Methods

Since the animal data suggested a variable toxic response in closely related species, ie, mouse and rat,[5] it was decided to treat the first three patients at different dose levels. If no biologic effects (ie, hematologic or gastrointestinal toxicity) or effects on tumor were noted, a rapid increase in dose to 12 or 24 mg/kg/wk was planned. All patients were to receive at least four doses at the stipulated level.

Bone marrow biopsy was performed in all patients entered in the study. The following studies were obtained three times a week from each patient:

(*a*) Hematologic studies: a complete blood count including a white blood cell differential.

(*b*) Hepatic function: bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase, lactic dehydrogenase, albumin, and globulin.

(c) Electrolytes: sodium, potassium, carbon dioxide, calcium, and phosphorus.

(*d*) Renal function: blood urea nitrogen (BUN), uric acid, creatinine, and urinalysis.

RESULTS

Determination of the Biologically Active Dose

Patients treated with 1.5, 3.0, and 6.0 mg/kg given iv weekly exhibited no biologic effect from this agent (table 2).

[5] Mihich, E., and Simpson, C. L. Toxicologic effects of tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (TEPA-132). An interim report. Unpublished data.

TABLE 1.—Patients participating in initial study of TEPA-132

Patient No.	Age, sex	Diagnosis	Previous therapy
1	29, F	Osteogenic sarcoma	Surgery
2	52, M	Adenocarcinoma, cecum	5-FU
3	52, M	Squamous cell carcinoma, lung	Radiation therapy
4	64, M	Carcinoma, bladder (transitional cell)	5-FU + radiation therapy
5	22, F	Osteogenic sarcoma	Surgery
6	34, F	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
7	64, M	Carcinoma, bladder (transitional cell)	5-FU + radiation therapy
8	55, M	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
9	62, M	Carcinoma, kidney	Radiation therapy
10	21, M	Granular cell carcinoma	Surgery
11	56, M	Carcinoma, kidney	Medroxyprogesterone acetate
12	69, M	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
13	65, M	Carcinoma, kidney	Medroxyprogesterone acetate
14	57, F	Carcinoma, kidney	Medroxyprogesterone acetate
15	54, F	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
16	60, M	Carcinoma, kidney	Medroxyprogesterone acetate
17	61, M	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
18	34, M	Hodgkin's disease	Methotrexate + vincristine + procarbazine + prednisone (MOPP), radiation therapy
19	48, M	Carcinoid, appendix	ThioTEPA, cyclophosphamide, vincristine
20	25, M	Malignant teratoma	Vincristine, cyclophosphamide, prednisone
21	31, F	Lymphocytic lymphosarcoma	Nitrogen mustard, prednisone, cyclophosphamide
22	68, M	Carcinoma, kidney	Medroxyprogesterone acetate
23	50, M	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone
24	62, M	Carcinoma, kidney	Medroxyprogesterone acetate + testosterone

TABLE 2.—Toxic effects during initial low-dose therapy with TEPA-132

Patient No.	Diagnosis	Dose	Wbc count (cells × 10^3)		Platelet count (cells × 10^3)		Hgb level (g/100 ml)		Gastrointestinal toxicity
			Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	
1	Osteogenic sarcoma	1.5 mg/kg/wk × 4	5.1	6.3	230	350	12.1	10.8	None
2	Carcinoma, cecum	1.5 mg/kg/wk × 4	6.2	5.6	300	320	11.0	10.0	None
3	Carcinoma, lung	3.0 mg/kg/wk × 4	5.1	4.6	200	180	10.2	10.3	None
4	Carcinoma, bladder	3.0 mg/kg/wk	8.9	7.6	250	360	11.0	11.4	None
5	Osteogenic sarcoma	6.0 mg/kg/wk × 5	7.4	8.8	330	360	12.0	12.2	None
6	Carcinoma, kidney	12.0 mg/kg/wk × 4	9.8	9.0	220	270	11.0	10.1	+*
7	Carcinoma, bladder	12.0 mg/kg/wk × 4	10.3	10.1	180	220	9.8	10.0	+

*+ = Nausea and vomiting 2–3 hours after infusion.

Patients given 12 mg/kg/wk had nausea and vomiting 2–3 hours after the infusion of TEPA-132. This reaction was similar to that noted with nitrogen mustard-type alkylating agents. Such treatment for 1 month resulted in no other biologic effects.

Four patients were given doses of 24 mg/kg/wk iv. After two doses all four patients exhibited significant hematologic depression (fig 2, table 3), but within 2–4 weeks after the initial dose hematologic recovery was complete. Four patients were given 20 mg/kg/wk (Patients Nos. 18, 19, 20, and 21) (table 1). A pattern of effects occurred which was similar to that noted after treatment with 24 mg/kg/wk. Moderate hematologic depression after the second weekly dose and one case of severe depression of white blood cells (wbc) and platelets developed in three patients in the 20 mg/kg/wk group. Again, recovery of all blood elements occurred 2–3 weeks after cessation of therapy.

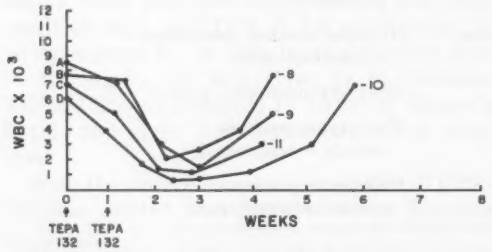

FIGURE 2.—The effect of TEPA-132 (24 mg/kg/wk for 2 doses) on the peripheral wbc count.

Nine patients were given 18 mg/kg of TEPA-132 iv every 2 weeks. The hematologic effects of this treatment regimen in six of nine patients are illustrated in figure 3. There was mild but consistent depression of wbc and platelet counts 1 week after each dose, but recovery usually occurred before the second dose was given. Platelet counts never fell to less than 100,000 cells/mm^3 in any patient. By the end of the tenth week six patients exhibited progression of their metastatic disease and they were removed from the study. No cumulative toxic effects were observed in this group.

Three patients continued to receive 18 mg/kg of TEPA-132 beyond 10 weeks (table 4, fig 4). Prolonged depression of wbc and platelet counts developed in one of these patients (No. 24) after his fifth dose. Recovery was slow, requiring 6 weeks for wbc and platelet counts to return to pretreatment levels. Two patients continue to receive TEPA-132 at the above dosage, and both are beyond their 14th week of therapy. In these patients complete hematologic

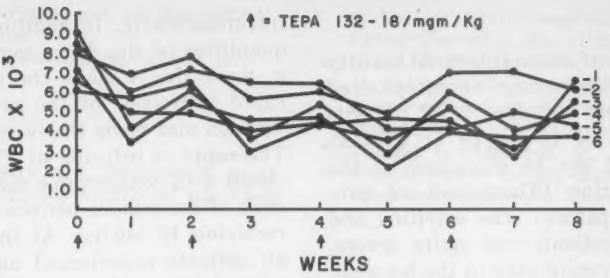

FIGURE 3.—The effect of TEPA-132 (18 mg/kg given every 2 weeks for 4 doses) on the peripheral wbc count.

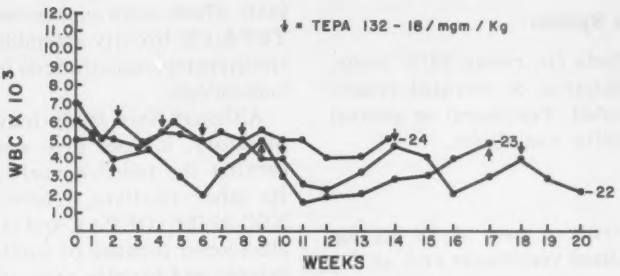

FIGURE 4.—The pattern of peripheral wbc response to prolonged administration of TEPA-132 at a dose of 18 mg/kg given every 2 weeks.

recovery now requires 3 weeks, rather than the 1–2-week period noted earlier in their therapeutic course. One of these patients has sustained an objective remission; in the other, metastatic disease seems to be unresponsive.

TABLE 3.—Effect of TEPA-132 (24 mg/kg/wk × 2) on peripheral platelet count

Patient No.	Time of platelet nadir (wks after 2nd dose)	Platelet count at nadir (cells/mm³)	Recovery time after nadir (wks)
8	2	60,000	2
9	1	80,000	2
10	3	50,000	2
11	3	25,000	3

TABLE 4.—Effect of prolonged TEPA-132 administration (18 mg/kg every 2 wks) on peripheral platelet count

Patient No.	Time of platelet nadir* (wks)	Platelets at nadir (cells/mm³)	Recovery time after last dose (wks)
22	14	80,000	2
23	12	70,000	2
24	10	55,000	5

*Time after beginning of therapy.

TOXICITY

Hematologic

A pattern of hematologic toxicity was noted during the various treatment courses already described. Anemia was not a consistent occurrence at any dose level but leukopenia and moderate thrombocytopenia were consistently observed with doses of 18 mg/kg given for 12 weeks and longer. None of the hematologic toxic effects were life threatening and there were no deaths attributable to the drug. Some evidence of cumulative toxicity was noted with prolonged administration of this agent. This was true in the one patient who had an objective remission as well as those who were in relapse or whose disease was unresponsive.

Gastrointestinal

The only evidence of gastrointestinal toxicity was the nausea and vomiting which occurred 2–3 hours after drug infusion in all patients who received doses of 12 mg/kg or greater. This reaction could not be abolished by the use of chlorpromazine (Thorazine) or prochlorperazine (Compazine). The vomiting and retching in some patients was quite severe, necessitating an overnight stay in the hospital. Stomatitis or pharyngitis did not occur, and there was no detectable hepatic impairment that could in any way be drug related.

Renal and Nervous System

No renal toxic effects (ie, rising BUN levels, urinary casts, proteinuria, or elevated creatinine levels) were noted. Peripheral or central nervous system toxicity was absent.

Regional Toxicity

All patients receiving a dose of 18 mg/kg had evidence of localized venospasm and, at the end of therapy, venous thrombosis at the site of infusion. Extravasation of TEPA-132 at this concentration caused erythemia, warmth, and pain but no slough. Peripheral thrombosis was extensive in patients receiving prolonged therapy and posed a major problem in finding suitable sites for future iv therapy.

DISCUSSION

These initial studies of TEPA-132 were designed to explore the clinical pharmacologic effects of this triethylenephosphoramide analog. A reasonably safe and biologically effective dose schedule was determined. A dose of 18 mg/kg given iv every 2 weeks produced mild hematologic toxicity, and in at least one case it produced objective remission of metastatic renal cell carcinoma. Prolonged administration of this agent may require dosage schedule modifications. An increasing period of hematologic depression was observed after 8 weeks of drug therapy, requiring, in some instances, a recovery period which lasted for 3 weeks after the last dose was given.

The iv route was used because of variable absorption when the drug was given orally or intramuscularly. In addition, the use of large quantities of the drug, sometimes exceeding 1 g, made the iv route the most practical. The rapid hydrolysis of the compound in an acid medium also made the iv route more desirable. The rapid iv infusion of TEPA-132 was associated with considerable venospasm and thrombosis of the smaller peripheral veins in patients receiving 18 mg/kg. At this therapeutic dose all patients experienced nausea and vomiting several hours after drug infusion. In several patients vomiting and retching were severe enough to require hospitalization for at least 48 hours. No other gastrointestinal or stomal toxic effects were encountered at any dose level. TEPA-132 toxicity resembled that reported for triethylenephosphoramide in that it was largely hematologic.

Although this study may be considered exploratory, it does give some indications concerning the possible usefulness of this agent. Its other relatives, triethylenephosphoramide, NSC-49421 (DEPA), and thioTEPA, have been abandoned because of limited therapeutic usefulness and toxicity compared to other alkylating agents (2). TEPA-132 may well share their fate.

LIST OF COMPOUNDS

Cyclophosphamide: NSC-26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; 5-FU

Medroxyprogesterone acetate: NSC-26386; pregn-4-ene-3,20-dione, 17-hydroxy-6α-methyl-, acetate; Provera

Methotrexate: NSC-740; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Meturedepa: NSC-51325; carbamic acid, [bis(2,2-dimethyl-1-aziridinyl)phosphinyl]-, ethyl ester

Nitrogen mustard: NSC-762; diethylamine, 2-2′-dichloro-*N*-methyl-, hydrochloride; HN2

NSC-49421: phosphinic amide, *P*,*P*-bis(1-aziridinyl)-*N*,*N*-diethyl-; DEPA

Prednisone: NSC-10023; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

TEPA-132: phosphine oxide, tris(2,2-dimethyl-1-aziridinyl)-

Testosterone: NSC-9700

ThioTEPA: NSC-6396; phosphine sulfide, tris(1-aziridinyl)-

Triethylenephosphoramide: NSC-9717; phosphine oxide, tris(1-aziridinyl)-; TEPA

Uredepa: NSC-37095; carbamic acid, [bis(1-aziridinyl)-phosphinyl]-, ethyl ester
Vincristine: NSC-67574; leurocristine, sulfate (1:)

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2. SYKES, M. P., KARNOFSKY, D. A., PHILIPS, F. S., ET AL. Clinical studies on triethylenephosphoramide and diethylenephosphoramide, compounds with nitrogen-mustard-like activity. Cancer 6:142–148, 1953.
3. FARBER, S., APPLETON, R., DOWNING, V., ET AL. Clinical studies on the carcinolytic action of triethylenephosphoramide. Cancer 6:135–141, 1953.

Evaluation of Cyclophosphamide (NSC-26271) and 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962) in the Treatment of Patients With Inoperable or Disseminated Lung Cancer [1,2]

David L. Ahmann, David T. Carr, Douglas T. Coles, and Richard G. Hahn [3]

SUMMARY

Forty-eight patients with nonlocalized bronchogenic carcinoma were randomly selected and treated with either 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or cyclophosphamide (or both). Five of the 48 patients (three who had received BCNU and two who had received cyclophosphamide) responded to initial treatment. Crossover treatment with the alternate drug after unsuccessful treatment with the initial drug resulted in no additional responses. Toxic effects included gastrointestinal upset and myelosuppression and, in general, were acceptable. There was one death after a course of BCNU that was attributable to the myelosuppressive effects of this agent. The medain survival time of the patients who died after treatment was 18 weeks, and the mean survival time was 15 weeks. Survival times were virtually the same for both treatment groups.
[Cancer Chemother Rep 56:401–403, 1972]

Presently available chemotherapeutic agents have not been consistently effective in palliating bronchogenic carcinoma; however, recent Veterans' Administration studies have shown a small but statistically significant increase in the survival time of patients with lung cancer who have been given parenteral doses of either cyclophosphamide (1) or BCNU.[4]

In the present study, a prospective randomized schedule of treatment with BCNU and cyclophosphamide was used in an effort to assess the clinical effectiveness of these two drugs (as indicated by objective measurable tumor response) in the management of unresectable or disseminated lung cancer.

MATERIALS AND METHODS

Patients with progressive unresectable lung cancer who were ineligible for present radiotherapeutic protocols because of the extent of their disease were included in this study. Patients who had had radiation therapy to the lung or previous cytotoxic chemotherapy with any agent other than BCNU or cyclophosphamide were admitted to the study if their indicator lesions were not within the previously irradiated field and if they fulfilled all other criteria for the study. Pathologic confirmation of the diagnosis was obtained for each patient. Each patient was maintaining a reasonable state of nutrition, and each had a hematologic status that did not preclude vigorous cytotoxic

[1] Received Nov 22, 1971; accepted Jan 27, 1972.

[2] Cyclophosphamide: 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. The authors wish to express appreciation to Dr. Stephen K. Carter, Chief, Cancer Therapy Evaluation Branch, Chemotherapy, National Cancer Institute, for his assistance and for providing the drugs used in this study.

[3] Mayo Clinic and Mayo Foundation, Rochester, Minn.

[4] Wolf, J. BCNU therapy of lung cancer. *In* Proceedings of the Chemotherapy Conference on the Chemotherapy of Solid Tumors: An Appraisal of 5-Fluorouracil and BCNU (Carter, S. K., ed). Bethesda, National Cancer Institute, 1970, pp 203–211.

chemotherapy, that is, each had a leukocyte count greater than 4000 cells/mm³ and a platelet count greater than 100,000 cells/mm³. All patients had measurable malignant lesions and adequate renal function, as demonstrated by normal concentrations of urea or creatinine in the blood. None of the patients had received treatment previously with either of the agents to be tested.

Each patient's disease was classified according to (*a*) cell type of bronchogenic carcinoma (squamous cell, adenocarcinoma, large cell, or small cell), (*b*) known duration (less than or more than 1 year from the time of diagnosis), and (*c*) evidence of extrathoracic visceral involvement (liver, bone, and brain) or confinement to intrathoracic structures and lymph nodes only (*see* table 1). Of the 48 patients in the study, 43 had had lung cancer for less than 1 year. None of the patients with extrathoracic metastatic disease had had lung cancer for more than 1 year. After type, duration, and extent of disease had been ascertained for patients in the study, they were assigned at random to one of two treatment programs. In one treatment program, BCNU was given by intravenous (iv) infusion (over 30 minutes) at an initial dose of 100 mg/m²/day for 3 days. After the first ten patients had been treated at this dose, the treatment dose was reduced to 75 mg/m²/day for 3 days. This change was made because of the undue hematologic toxicity which had occurred with the higher dose. In the other treatment program, cyclophosphamide was given by rapid iv infusion at a dose of 10 mg/kg of ideal weight on each of 3 successive days. After each course of treatment, leukocyte counts and platelet counts were obtained several times weekly for either 4 weeks (when cyclophosphamide had been given) or 8 weeks (when BCNU had been given).

Re-evaluation at 4 weeks and again at 8 weeks included case history and physical examination and measurement of indicator lesions. Patients in whom either objective improvement or no progression of disease was noted were retreated with the same agent; for these patients, the dose schedule was adjusted to preclude undue toxicity. Patients in whom metastatic disease had progressed during initial treatment were treated subsequently with the alternate drug. Secondary treatment was not begun for patients in either group, however, until after an interval of either 4 weeks (for patients who had received cyclophosphamide initially) or 8 weeks (for patients who had received BCNU initially).

TABLE 1.—Distribution of patients with bronchogenic carcinoma who were treated with BCNU or cyclophosphamide

Factor	BCNU (25 patients)	Cyclophosphamide (23 patients)
Type of carcinoma		
Squamous cell	6	6
Small cell	4	5
Adenocarcinoma	8	8
Large cell	7	4
Duration		
<1 yr	20	22
≥ 1 yr	5	1
Involvement		
Thoracic and nodal	11	8
Extrathoracic	14	15

Objective improvement was defined as a measurable reduction in the size of the indicator lesion by at least 33% of the product of the perpendicular diameters. Patients were considered to have progressive disease if any lesion increased in size or if any new lesions developed.

RESULTS

The therapeutic results were not impressive (table 2). Five of the 48 patients responded: three to BCNU and two to cyclophosphamide. In each of these five patients, the response occurred after the initial course of treatment. Both of the patients who responded to treatment with cyclophosphamide had small cell carcinoma. Of the three patients who responded to BCNU, one had squamous cell carcinoma, one had small cell carcinoma, and one had large cell carcinoma. The responses to BCNU lasted, respectively, 5, 5, and 6 months, and the responses to cyclophosphamide lasted, respectively, 7 and 10 months. The median survival time of the 37 patients who have died thus far after treatment was 18 weeks and the mean was 15 weeks. Length of survival was virtually the same for both treatment groups.

TABLE 2.—Objective responses of patients with bronchogenic carcinoma to treatment with BCNU and cyclophosphamide

Treatment	No. of responding patients receiving— BCNU	Cyclophosphamide
Primary	3 of 25	2 of 23
Secondary	0 of 8	0 of 8
Total	3 of 33	2 of 31

TOXICITY

The initial BCNU dose of 100 mg/m²/day for 3 days was changed to 75 mg/m²/day for 3 days because excessive leukopenia and thrombopenia had occurred with the higher dose. Toxicity occurring after this reduction in treatment dose was, for the most part, acceptable (table 3).

The death of one patient was attributable to the use of BCNU; in this patient, leukopenia and sepsis had occurred. Fifteen other patients died within 8 weeks after the beginning of treatment: nine of these patients had received BCNU and six had received cyclophosphamide. Death was probably related to the severity of illness in these patients, although systemic chemotherapy may have added to their difficulties.

A few patients who received large doses of BCNU complained of pain (often associated with mild phlebitis) along the site of iv injection. This effect was circumvented for these patients by diluting the BCNU with a larger volume of glucose and water.

Gastrointestinal toxic effects of treatment included nausea and vomiting only, and none of the patients had symptoms severe enough to warrant the use of iv administered fluids.

TABLE 3.—Toxic effects of treatment with BCNU and cyclophosphamide in patients with bronchogenic carcinoma

Toxic effect	Treatment group BCNU* (33 patients)	Cyclophosphamide (31 patients)
Gastrointestinal (nausea and vomiting)	10	23
Leukopenia (< 3000 cells/mm³)	12	7
Thrombopenia (< 100,000 cells/mm³)	16	1
Alopecia	0	3

*One death attributable to treatment with the drug.

COMMENT

Bronchogenic carcinoma is not very responsive to the various cytotoxic chemotherapeutic agents available at present. Although survival time in some studies seems to be slightly increased by the use of these agents, they have not been of much clinical value. In this study, neither BCNU nor cyclophosphamide showed enough antitumor activity to warrant consideration for routine use in the treatment of bronchogenic carcinoma.

REFERENCE

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Absorption, Distribution, and Excretion of 5-Azacytidine (NSC-102816) in Man [1,2,3]

W. M. Troetel,[4] A. J. Weiss,[5] J. E. Stambaugh,[4,5] J. F. Lauclus,[5,6] and R. W. Manthei [4]

SUMMARY

Patients with advanced cancer were given 5-azacytidine labeled at position 4 with radioactive carbon (^{14}C) by either the intravenous (iv) or subcutaneous (sc) route. Absorption of the drug from the sc injection site was rapid and peak plasma levels were attained within one-half hour. Within 2 hours, the plasma level of radioactivity was approximately equal to that noted in the patients treated iv. The plasma half-life after iv injection was 3.5 hours; after sc administration, the plasma half-life was 4.2 hours. Patients receiving the drug sc excreted less drug in the urine than did those receiving the drug iv. No radioactivity was detected in the expired carbon dioxide when the drug was given by either route. Drug uptake into tumor tissue was always greater than uptake into surrounding normal tissue. The highest concentrations of radioactivity in the tissues were achieved when the drug had been given iv. Traces of radioactivity were still detectable in the tissues for as long as 6 days after administration of the drug. The incorporation of radioactivity into tumor RNA but not into DNA was demonstrated. The maximum level of radioactivity detected in the spinal fluid was equivalent to 0.2 μg of 5-azacytidine per milliliter of fluid.

[Cancer Chemother Rep 56:405–411, 1972]

The cytidine analog, 5-azacytidine, was synthesized by Pískala and Šorm (1) and was shown to be a potent inhibitor of lymphoid leukemia in the AK strain of mice (2). 5-Azacytidine also exhibited pronounced cancerostatic effects in vivo against L1210 leukemia and T4 lymphoma in mice (3) and against Ehrlich ascites tumor in mice (4). Li and coworkers, using the L1210 leukemia system, established that the drug inhibited the incorporation of tritiated thymidine or deoxyadenosine into DNA to a greater extent than it inhibited the incorporation of tritiated uridine into RNA (5). The antimetabolite may act to disrupt protein synthesis by incorporating into messenger RNA, thereby interfering with the translocation process of protein synthesis (6,7).

Studies in mice have indicated that 5-azacytidine tends to concentrate in lymphatic tissues after intraperitoneal (ip) administration and is rapidly excreted as unchanged drug and as metabolites in the urine (8,9). In the present

[1] Received Dec 15, 1971; revised Feb 16, 1972.

[2] Supported by grant CA–06071 and contract NIH–70–2136 from Chemotherapy, National Cancer Institute (NCI), National Institutes of Health.

[3] 5-Azacytidine: *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-. Supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, NCI.

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[6] Supported by Public Health Service Research Fellowship No. 1–F03–CA–52030–01, from the Department of Health, Education, and Welfare.

study, patients with advanced cancer were given 5-azacytidine labeled at position 4 with radioactive carbon (5-azacytidine-4-^{14}C) by either the intravenous (iv) or subcutaneous (sc) route. A comparison of the absorption, distribution, and excretion of the radioactive material in the two treatment groups was made. The incorporation of the antimetabolite into tumor nucleic acids was also investigated.

MATERIALS AND METHODS

^{14}C-labeled 5-azacytidine (fig 1) with a specific activity of 49.1 millicuries/millimol (200 microcuries/mg) was synthesized by the Monsanto Research Corporation, Dayton, Ohio. A radiochemical purity of greater than 98% was confirmed by a radioscan of a chromatogram developed in a system of *n*-butanol saturated with water. The labeled material was stored in a desiccator at −10 C until just prior to use.

Unlabeled 5-azacytidine was mixed with approximately 85 microcuries of radioactive drug and was administered to 11 patients at the doses indicated in table 1. The drug was reconstituted with bacteriostatic water for injection and administered by either the iv or sc route through a Millipore Swinnex-25 disposable sterilized filter (0.22μ). Appropriate informed consent was obtained from all patients treated with the labeled drug.

All patients selected for treatment had normal liver and kidney function and no other coexisting disorder, such as an infectious disease process, was present. Blood specimens were collected at the times indicated in table 2 into

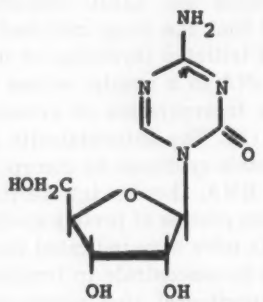

FIGURE 1.—Structure of 5-azacytidine; * indicates position of ^{14}C.

10-ml heparinized glass vacuum tubes. An aliquot of whole blood (0.1 ml) was assayed for radioactivity by combustion in a Packard Tissue Oxidizer (Model 305). The remainder of the blood was centrifuged and the plasma was separated from the packed cell fraction. Plasma radioactivity (0.1-ml sample) was counted in a Packard Liquid Scintillation Spectrometer (Model 3375). A fluor of the following formula was used: 1,4-bis-(2-[5-phenyloxazolyl])-benzene (POPOP), 99 mg; 2,5-diphenyloxazole (PPO), 5.0 g; and Triton X–100 (Packard), 330 ml; diluted with toluene to a total volume of 1000 ml.

Urine was collected at specific intervals for 48–72 hours, the total volume was recorded, and 0.1-ml portions were analyzed for radioactivity in the Triton-X-100-containing fluor described previously. One sample of cerebrospinal fluid (CSF) was obtained from each of four patients at different times and 0.1 ml of each sample was assayed for radioactivity. Samples of ascitic fluid were obtained from only one patient. In all instances, quench corrections were made with an external standard.

Levels of radioactivity in expired carbon dioxide (CO_2) were determined by having patients exhale for 5-minute periods into redistilled ethanolamine. The ethanolamine (4.5 ml) was mixed with 2 volumes of methanol and radioactivity was counted by the addition of 1 volume of fluor composed of PPO, 15 g; *p*-bis-(*o*-methyl-styryl)-benzene (bis-MSB), 1 g; diluted to 1000 ml with toluene. For this assay, counts were corrected by the efficiencies determined with an internal standard of ^{14}C-labeled toluene. Also, CO_2 was collected for 48 hours from male Wistar rats (150–200 g) who had received iv or sc injections of ^{14}C-labeled 5-azacytidine (15 mg/kg, 2 microcuries/animal) in water, and placed in a Nuclear Associates Metabolism Chamber (Model CR 350). The trapping agent for CO_2 was ethanolamine and aliquots (4.5 ml) were assayed for radioactivity as previously described.

For fecal excretion studies, rats received iv or sc injections of labeled 5-azacytidine (15 mg/kg, 2 microcuries/animal) and were housed in individual metabolism cages which permitted the separate collection of urine and feces. The rats were given food and water prior to

TABLE 1.—Patients treated with 5-azacytidine-4-^{14}C

Patient No.	Sex	Age	Weight (kg)	Route of drug administration	Diagnosis
1	F	56	70.5	Iv	Metastatic cystadenocarcinoma of the ovary
2	M	58	75.9	Iv	Metastatic adenocarcinoma of the colon and metastatic melanoma of the retromolar area and buccal mucosa
3	F	57	73.2	Iv	Metastatic cystadenocarcinoma of the ovary
4	F	64	52.3	Iv	Metastatic adenocarcinoma of the breast
5*	M	47	50.5	Iv	Metastatic adenocarcinoma of the rectum
6	M	57	61.4	Iv	Metastatic adenocarcinoma of the colon
7	M	67	54.1	Sc	Metastatic adenocarcinoma of the colon
8	F	45	40.0	Sc	Metastatic adenocarcinoma of the stomach
9	F	63	33.2	Sc	Metastatic squamous cell carcinoma of the lung
10	F	41	89.1	Sc	Metastatic adenocarcinoma of the colon
11	F	38	86.4	Sc	Metastatic synovial sarcoma of the knee

*Received 0.4 mg/kg. All other patients received 1.6 mg/kg.

TABLE 2.—Plasma concentration of 5-azacytidine-4-^{14}C in patients after iv or sc treatment*

Time after administration	Concentration after iv administration in patient No.—							Concentration after sc administration in patient No.—			
	1	2	3	4	5	6	Mean ± SD	7	8	9	Mean ± SD
2 min	11.9	5.74	11.2	—	13.8	4.82	9.49 ± 3.55	—	—	—	—
10 min	2.83	3.44	4.62	4.11	6.64	2.89	4.09 ± 1.31	0.42	—	0.53	0.48
20 min	2.42	—	3.95	3.15	2.60	2.54	2.93 ± 0.60	—	—	—	—
30 min	2.20	2.83	3.54	3.03	2.60	2.13	2.72 ± 0.49	—	2.15	—	—
1 hr	1.91	2.29	3.48	2.65	2.40	1.70	2.41 ± 0.57	1.45	2.07	1.72	1.75 ± 0.25
2 hrs	2.09	1.74	2.61	1.67	1.92	1.41	1.91 ± 0.38	1.37	1.50	1.83	1.57 ± 0.19
4 hrs	1.39	1.24	2.03	—	1.24	1.07	1.39 ± 0.33	1.05	1.22	1.38	1.22 ± 0.14
6 hrs	1.07	0.83	—	—	—	0.98	0.96 ± 0.10	0.87	0.79	—	0.83
12 hrs	0.59	0.30	0.87	0.28	0.30	0.90	0.54 ± 0.27	0.47	0.49	0.55	0.50 ± 0.03
24 hrs	0.29	0.09	0.40	0.08	0.07	0.24	0.20 ± 0.12	0.17	0.31	0.19	0.22 ± 0.06
48 hrs	0.08	—	0	—	—	—	0.04	0.07	0.02	—	0.05

*Values expressed as μg equivalents of 5-azacytidine/ml of plasma.

injection, but after the injection had been given, food but not water was withheld. Feces were collected for 24 and 48 hours after injection. The collected feces were homogenized and weighed, and an aliquot was combusted as described previously and assayed for radioactivity.

Surgically obtained samples of tissue from patients were immediately frozen in glass tubes immersed in a dry ice-acetone bath. Tissues were thawed, rinsed in cold saline, and blotted dry, and total radioactivity was determined by combustion. Uptake of radioactivity into RNA and DNA was determined by the procedure of Wannemacher and co-workers (10). Since the level of radioactivity in the tissue samples was low (*see* table 4), samples were assayed in a liquid scintillation counter preset for 50 minutes or 1000 counts. This setting afforded a standard error in net counting rate of approximately 2%–5%.

RESULTS AND DISCUSSION

Blood

The plasma concentration of radioactivity, expressed as microgram (μg) equivalents of ^{14}C-labeled 5-azacytidine per milliliter of plasma, is shown in table 2. The term "μg equivalent" is defined as the level of radioactivity resulting from drug and metabolites which is equivalent to 1 μg of unchanged 5-azacytidine. The data from patients to whom the drug was given iv (table 2) indicate a triphasic disappearance of radioactivity from plasma. The half-life determined from 30 minutes to 6 hours after iv injection was 3.5 hours. This compared with a half-life of 4.2 hours after sc injection for the same time period. It can be calculated from the data of Raška and co-workers that the half-life of 5-azacytidine and radioactive metabolites in mice is 3.8 hours (8). Thus, there is good correlation between the data reported for mice and the plasma half-life observed in patients. There is no significant difference in plasma levels of radioactivity in patients 2–48 hours after injection regardless of the route of administration. It can be seen also that absorption from the sc site is rapid since a peak level of radioactivity (2.15 μg/ml) was attained in one patient within one-half hour after injection.

Although it was not possible in these studies to determine whether absorption from the sc site in patients was complete, in rats given sc injections of the labeled drug, less than 1% of the administered radioactivity remained at the injection site 2 hours after administration. This was determined by combustion of the skin and subcutaneous tissue from the injection site. Thus, it is likely that the drug is both rapidly and completely absorbed after sc administration.

Ascitic and Cerebrospinal Fluids

Samples of ascitic fluid were obtained from patient No. 1 at half-hour intervals for 6 hours after iv administration of 5-azacytidine. Drug was detectable in the ascitic fluid as early as one-half hour after administration, but did not reach equilibrium with the plasma (1.1 μg/ml) until 6 hours later.

Table 3 indicates that the level of radioactivity in spinal fluid was low when compared with plasma levels. The maximum level of radioactivity in the spinal fluid was noted 24 hours after treatment when the plasma level had declined to 0.4 μg/ml. Since the levels of radioactivity in the CSF were too low to permit identification of the products present, it was not possible to determine whether unchanged drug passed into the spinal fluid. From the limited data available, however, it does appear that significant levels of drug are not attainable in the central nervous system.

Excretion Studies

Figure 2 illustrates the cumulative percent of urinary excretion of radioactivity following iv and sc administration of 5-azacytidine. There was significantly greater excretion of radioactivity over a 48-hour period after iv administration than after sc therapy (85% vs 50%). This was correlated with a higher renal clearance rate in the patients treated iv. Renal clearance of radioactivity was determined in four patients with known normal renal function. Two patients treated iv had renal clearance rates of 76.3 and 127.6 ml/minute and two patients treated sc had renal clearance rates of

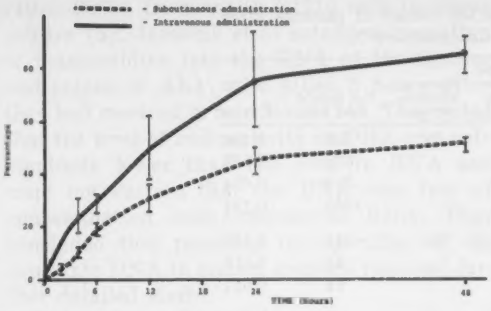

FIGURE 2.—Cumulative percent excretion of urinary radioactivity after the iv and sc administration of 1.6 mg/kg 5-azacytidine to patients. Each point represents the mean ± SD (determined from data for 3–5 patients).

50.4 and 55.2 ml/minute. Since the rate of clearance of radioactivity in all four patients was less than the average glomerular filtration rate of 130 ml/minute, it is apparent that active tubular secretion of labeled substances does not occur (11).

Expired CO_2 from patients was not radioactive regardless of the route of administration of the drug. Forty-eight-hour collections of CO_2 from rats treated iv or sc also were not radioactive. However, any CO_2 produced by the metabolism of 5-azacytidine would probably result from the production of ribosylbiuret (1). Since metabolism to this product would cause the loss of the carbon atom at position 6 of the triazine ring, it would not be detected with the drug labeled at position 4 as in this study.

The rat was used for the determination of the fecal excretion of the drug. Only 3% of the total injected radioactive dose was detected in feces over a 48-hour period after either route of administration. In future studies in patients, the fecal excretion of the drug will be compared with that noted in rats.

Tissue Studies

Levels of radioactivity detected in the tissues of six patients are presented in table 4. The tissue radioactivity is expressed as dpm/g (wet weight) and as the F value as defined in table 4. The latter value permits a comparison of the level of activity in the tissues of patients receiving various doses of radioactive material (12). The data indicate that administration of the drug by the iv route leads to greater tissue uptake and retention of the drug. This is based on the data from samples of carcinoma of the colon from patients Nos. 2, 6, and 10. Specimens of colon from the first two patients treated iv had F values of 0.136 and 0.133 and tissue from the colon of patient No. 10 (treated by the sc route) had an F value of 0.064. These samples of colon were all obtained 44–52 hours after administration of the drug.

In four patients (Nos. 2, 6, 7, and 11), when the carcinoma was removed, adjacent normal tissue was also removed for study. In each case there was a higher uptake of drug in tumor tissue than in the surrounding normal tissue as indicated by the F values in table 4. In patient No. 2, the carcinoma had about 1.3 and 2.5 times the level of radioactivity found in the normal lymph node and appendix respectively. The carcinoma of the colon of patient No. 6 had 1.5 times the radioactivity of normal intestine. Assay of the carcinoma of the lung of patient No. 11 indicated that 2.2 times as much

TABLE 3.—Cerebrospinal fluid (CSF) and plasma levels of radioactivity in patients after iv administration of 5-azacytidine-4-^{14}C

Patient No.	Time after administration (hrs)	CSF*	Plasma*	Plasma-CSF ratio
2	0.75	0.03	2.56	85.3
1	2.0	0.12	1.69	14.0
5	4.5	0.12	1.24	10.3
3	24.0	1.20	0.40	0.33

*Values expressed as μg equivalents of 5-azacytidine-4-^{14}C/ml of spinal fluid and plasma; plasma sample obtained from each patient at same time as spinal fluid sample.

TABLE 4.—Retention of radioactivity in the tissues of patients after iv or sc treatment with 5-azacytidine-4-^{14}C

Patient No.	Tissue	Time after administration (hrs)	Route of administration	Net dpm/g (wet wt)	F value*
2	Carcinoma of the colon	48	Iv	798	0.136
	Normal lymph node			629	0.107
	Normal appendix			319	0.054
	Normal liver			1062	0.181
7	Carcinoma of the colon	30	Sc	416	0.152
	Carcinoma of the colon	144		45	0.016
	Normal intestine			31	0.011
9	Carcinoma of the lung, subcutaneous metastatic thigh lesion	24	Sc	410	0.082
10	Carcinoma of the colon	48	Sc	178	0.064
6	Carcinoma of the colon	48	Iv	371	0.133
	Normal intestine			252	0.090
11	Carcinoma of the lung	48	Sc	170	0.068
	Normal lung			78	0.031
	Normal liver			168	0.067

*F value = $\frac{\text{dpm/g tissue (wet wt)}}{\text{dpm administered per g body wt}}$.

radioactivity had concentrated in the tumor as compared with normal lung. Six days after drug administration, analysis of surgically obtained specimens of the carcinoma of the colon as well as of normal intestine from patient No. 7 indicated that traces of the drug were still retained. The level of radioactivity was low, but the activity in the tumor was approximately 1.5 times greater than that in normal intestine. Thus, regardless of the route of administration, 5-azacytidine tended to concentrate in tumor tissue. When the same types of tumor were compared, however, a higher level of drug uptake after iv administration was evident.

Uptake into the DNA of tumor tissue could not be demonstrated, but incorporation of radioactivity into the RNA was evident as indicated in table 5. The extent of incorporation into RNA ranged from 0.88 to 2.50 dpm/μg RNA. It is of interest to note that the highest specific activity in RNA was determined in patient No. 9 from whom tissues had been obtained 24 hours after injection of 5-azacytidine. The two other patients (Nos. 2 and 6) were treated surgically 48 hours after injection.

Zadražil et al have demonstrated incorporation of 5-azacytidine into the DNA of *E. coli* (13). Li and co-workers reported that 5-azacytidine was incorporated into polynucleotides of the liver, spleen, and ascitic cells of leukemic mice, and into both the RNA (80%–90% total incorporated radioactivity) and the DNA

TABLE 5.—Distribution of radioactivity in tumor after administration of 5-azacytidine-4-^{14}C

Patient No.	Radioactivity in 10% TCA acid-soluble fraction (%)	Radioactivity in RNA (%)	Specific activity of RNA (dpm/μg RNA)	Radioactivity in DNA
2	27.6	77.0	1.18	ND*
6	86.0	18.0	0.88	ND
9	57.0	37.6	2.50	ND

*ND = no radioactivity detectable.

(10%–20%) fractions in L1210 cells in tissue culture (5). Jurovčík et al noted incorporation of 5-azacytidine into the DNA of the thymus and spleen of AKR mice killed 3 hours after they had received ip injections (14). They noted that the level of radioactivity in DNA was substantially lower than the level in RNA and were not certain that the DNA was free of contamination with radioactive RNA. They concluded that proof of incorporation of the drug into DNA in animal systems required further detailed study.

Although we did not detect incorporation of the drug into the DNA of tumor tissue in the patients studied, it is still possible that this may have occurred. Incorporation could occur rapidly and could be followed by disruption of the DNA prior to the 24- and 48-hour sampling periods we used. Future studies of patients from whom tissue can be obtained 2–4 hours after the administration of 5-azacytidine would resolve the problem of whether there is incorporation into DNA followed by a rapid breakdown of the triazine ring. Such samples would also indicate more clearly even minimal incorporation because they would have higher levels of radioactivity.

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Phase I Study of 5-Azacytidine (NSC-102816) [1,2,3,4]

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SUMMARY

Thirty patients with various solid tumors were treated with 5-azacytidine. Total doses ranged from 1.0 to 24.0 mg/kg and were given over a minimal period of 8 days. The major toxic effect was hematologic with significant leukopenia and thrombocytopenia usually occurring 20–30 days after the start of therapy, especially at higher dose levels. The marrow depression lasted 1–5 weeks and was fully reversible. Nausea and mild diarrhea were common following injection of the drug. Serum glutamic oxaloacetic transaminase levels rose in several patients. No other evidence of hepatic toxicity was seen. Objective remissions were noted in seven of 11 patients with cancer of the breast, two of five with melanoma, and two of six with cancer of the colon.

[Cancer Chemother Rep 56:413–419, 1972]

5-Azacytidine is an analog of cytidine in which nitrogen is substituted for the carbon in the 5 position of the pyrimidine ring. It was first synthesized by Píškala and Šorm in 1964. It is produced microbiologically by a species of Streptoverticillium, *S. Ladakanus*. It is a white crystalline material which is quite soluble in water and various organic solvents. Its formula is $C_8H_{12}N_4O_5$ (1,2).

Studies with the compound reveal it to be active against several animal neoplasms, including the L1210 tumor (3,4). It was relatively inactive, however, against the Walker 256 tumor. The compound was incorporated into plant and bacterial RNA and DNA and inhibited nucleic acid and protein synthesis (5,6). It is effective in causing chromosomal abnormalities in plants (7). Apparently, several patients have been treated in Europe; however, no published data on the results of the Czechoslovakian clinical studies have been available to date.

In animal experiments the efficacy of the drug as an antitumor agent was highly schedule-dependent, the most effective schedule being a daily dose given over 10 days.[7] The maximum tolerated dose in the most sensitive animal, the beagle, was 0.55 mg/kg/day given over a 5-day period.[7] Since data from animal studies seemed to indicate that a 10-day schedule would be proper for initial clinical use, patients in our study received beginning total daily doses of 0.03 mg/kg for 10 days.

MATERIALS AND METHODS

The drug was supplied in 50-mg vials. When the drug is to be administered, it is dissolved

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[2] Supported by grant CA–06071 and contract NIH–70–2136 from Chemotherapy, National Cancer Institute (NCI), National Institutes of Health, Public Health Service.

[3] 5-Azacytidine: *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-. Supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, NCI.

[4] Performed as a phase I study for the Central Oncology Group (Dr. Robert O. Johnson, University of Wisconsin, Chairman).

[5] Supported by Public Health Service Research Fellowship No. 1F03-CA-52030–01 from the Department of Health, Education, and Welfare.

[6] Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pa.

[7] Clinical brochure: 5-azacytidine (NSC-102816), Oct 1970, 25 pp. Prepared by the NCI, Bethesda, Md.

in an appropriate volume of distilled water and then given immediately. In this study, when the drug was given intravenously (iv), it was given as rapidly as possible. Two patients were treated at each dose level. A modified Fibonacci scheme was used to determine dose increments. After toxicity occurred, dose increments were limited to 10% of the previous dose. To be eligible for the study, patients were required to have a white blood cell count (wbc) of 5000 cells/mm^3 or greater and a platelet count of 140,000 cells/mm^3 or greater. The blood urea nitrogen (BUN) value had to be less than 12 mg/100 ml and the bilirubin value less than 1.2 mg/100 ml. Further contraindications for therapy included radiation to the pelvis, chemotherapy in the 4-week interval prior to therapy, prior adrenalectomy or hypophysectomy, or life expectancy of less than 3 months. Complete blood cell counts and platelet counts were done at least twice weekly and blood chemistry tests were done once weekly.

The criteria for objective remissions were those of the Central Oncology Group, that is, a 50% or greater reduction in the area of the tumors being measured as approximated by a 50% reduction in the product of the major diameter of the tumor and its perpendicular. The regression must have lasted for a minimal period of 4 weeks with no new lesions appearing during this time. All lesions were measured weekly.

RESULTS

Tables 1 and 2 present a summary of the drug toxicity observed in the 30 patients treated. As can be seen, mild hematologic depression was noted at total doses of 12–16 mg/kg, and rather severe hematologic depression was noted in most patients receiving total doses of more than 16 mg/kg. The diarrhea noted was transient, occurring 2–3 hours after each injection; it was not disabling. In this series, we did not see stomatitis and dermatitis was rare. Hepatic function changes are found in animals treated with 5-azacytidine. Several of our patients had reversible rises in serum glutamic oxaloacetic transaminase (SGOT) levels, but no other hepatic abnormalties were noted. Although the hematologic depression was at times quite severe, serious infection occurred in only one patient and no patients died from this complication. Nausea almost invariably occurred after drug administration.

TABLE 1.—Hematologic toxicity

Patient No.	Sex	Dose[a]	Leukopenia			Thrombocytopenia		
			Nadir count (× 10^3 cells/mm^3)	Day nadir occurred	Day count normal	Nadir count (× 10^3 cells/mm^3)	Day nadir occurred	Day count normal
1	F	0.55 mg/kg, OD × 10	3.8	12	18	116	14	18
2	F	0.5 mg/kg, OD × 15	3.8	36	40	118	25	29
3	M	0.75 mg/kg, OD × 11	None	—	—	118	14	17
4	F	1.1 mg/kg, OD × 10	None	—	—	112	23	30
5	M	1.2 mg/kg, OD × 10	3.55	22	33	116	19	22
6	F	1.4 mg/kg, OD × 10	3.9	27	30	None	—	—
7	F	1.0 mg/kg, OD × 15	3.5	18	23	112	10	55
8	F	1.0 mg/kg, OD × 15	0.85	28	56	98	31	48
9	F	1.0 mg/kg, OD × 15	3.1	29	45	42	32	62

TABLE 1.—Hematologic toxicity—Continued

Patient No.	Sex	Dose*	Leukopenia			Thrombocytopenia		
			Nadir count ($\times 10^3$ cells/mm^3)	Day nadir occurred	Day count normal	Nadir count ($\times 10^3$ cells/mm^3)	Day nadir occurred	Day count normal
10	F	1.0 mg/kg, OD × 15	1.9	29	51	84	26	48
11	F	1.6 mg/kg, OD × 10	2.6	11	60	66	22	32
12	M	1.6 mg/kg, OD × 10	2.2	20	65	74	20	70
13	F	1.6 mg/kg, OD × 10	2.4	22	28	104	18	25
14	F	1.8 mg/kg, OD × 10	1.8	27	40	98	30	43
15	F	1.8 mg/kg, OD × 10	1.1	31	45	106	5	26
16	M	1.8 mg/kg, OD × 10	3.5	17	21	96	24	31
17	M	2.0 mg/kg, OD × 10	4.3	18	20	73	20	Died Day 20
18	M	2.0 mg/kg, OD × 10	0.5	18	Abnormal until Day 48	None	—	Died Day 48
19	F	2.0 mg/kg, OD × 10	None	—	—	88	49	56
20	F	2.0 mg/kg, OD × 10	1.2	22	42	48	28	42
21	F	2.0 mg/kg, OD × 10	0.95	23	72	46	20	55
22	F	2.0 mg/kg, OD × 10	2.9	32	37	90	11	77
23	F	2.0 mg/kg, OD × 10	3.3	23	68	96	54	86
24	M	2.0 mg/kg, OD × 10	1.0	28	50	52	31	53
25	M	2.2 mg/kg, OD × 10	0.9	29	39	32	17	27
26	F	2.2 mg/kg, OD × 10	0.25	23	57	38	23	54
27	F	2.4 mg/kg, OD × 10	1.0	26	50	116	26	28

*OD = daily dose.

Table 3 presents the initial remissions seen in this series and the relationship of dose to remission. Because parallel pharmacologic studies were being conducted (8), the patients selected for this study fell into relatively limited patterns of disease. Almost all patients treated who had either breast cancer or melanoma had soft tissue disease as their primary manifestation of cancer. In the group of patients with breast cancer, most had either chest wall recurrences or unresectable tumors involving the breast, while most of the patients with melanoma had multiple, subcutaneous, metastatic disease. In either group, lesions were measurable. All objective remissions were partial; no complete remissions were seen.

TABLE 2.—Non-hematologic toxicity*

Patient No.	Sex	Dose†	Degree of—				Rise in SGOT value (units)	
			Nausea	Diarrhea	Stomatitis	Dermatitis	From	To
28	F	0.55 mg/kg, OD × 10	0	0	0	0		
1	F	0.55 mg/kg, OD × 10	3	2	1	0		
2	F	0.5 mg/kg, OD × 15	2	1	1	0	73.5	174
3	M	0.75 mg/kg, OD × 11	0	0	0	0		
4	F	1.1 mg/kg, OD × 10	2	1	0	0		
5	M	1.2 mg/kg, OD × 10	1	1	0	0	14.0	30.0
29	F	1.2 mg/kg, OD × 10	0	0	0	0		
30	F	1.2 mg/kg, OD × 11	0	0	0	0		
6	F	1.4 mg/kg, OD × 10	0	0	0	0		
7	F	1.0 mg/kg, OD × 15	2	0	0	0	16	40.0
8	F	1.0 mg/kg, OD × 15	3	1	0	0	40	168
9	F	1.0 mg/kg, OD × 15	2	1	0	0	26.5	86.5
10	F	1.0 mg/kg, OD × 15	2	2	1	1		
11	F	1.6 mg/kg, OD × 10	2	0	0	0		
12	M	1.6 mg/kg, OD × 10	1	1	0	0		
13	F	1.6 mg/kg, OD × 10	1	2	0	0		
14	F	1.8 mg/kg, OD × 10	2	1	0	0	20	95.0
15	F	1.8 mg/kg, OD × 10	1	0	0	0		
16	M	1.8 mg/kg, OD × 10	1	1	0	0		
17	M	2.0 mg/kg, OD × 10	2	0	0	0		
18	M	2.0 mg/kg, OD × 10	3	2	1	0	47.5	142
19	F	2.0 mg/kg, OD × 10	1	1	0	0		
20	F	2.0 mg/kg, OD × 10	2	1	1	1		
21	F	2.0 mg/kg, OD × 10	2	1	0	0	75	144
22	F	2.0 mg/kg, OD × 10	3	1	1	0	26.5	96.0
23	F	2.0 mg/kg, OD × 10	2	1	0	0		
24	M	2.0 mg/kg, OD × 10	2	1	0	0	12.5	29.5
25	M	2.2 mg/kg, OD × 10	2	2	0	0		
26	F	2.2 mg/kg, OD × 10	2	2	0	0		
27	F	2.4 mg/kg, OD × 10	2	1	0	0		

*0 = No toxicity; 1 = mild toxicity; 2 = moderate toxicity; 3 = incapacitating toxicity.
†OD = daily dose.

The data suggest that 5-azacytidine may be useful in the treatment of the soft tissue metastatic disease of breast cancer and malignant melanoma. One significant remission and one borderline remission were seen in patients with cancer of the colon.

Several of the patients with breast cancer who had objective remissions had received prior therapy with 5-fluorouracil (5-FU) or cytosine arabinoside or both and were resistant to both.[8] Thus, there did not appear to be any cross resistance to 5-azacytidine in patients treated unsuccessfully with other available antipyrimidines.

The length of the remission obtained is not

[8] 5-Fluorouracil: NSC-19893.
Cytosine arabinoside: NSC-63878; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

TABLE 3.—Response to treatment with 5-azacytidine

Disease	Patient No.	Sex	Treatment*	Response†‡
Cancer of the breast	1	F	0.55 mg/kg, OD × 10	S–O
	4	F	1.1 mg/kg, OD × 10	S–O
	6	F	1.4 mg/kg, OD × 10	O
	7	F	1.0 mg/kg, OD × 15	P
	9	F	1.0 mg/kg, OD × 15	P
	10	F	1.0 mg/kg, OD × 15	P
	15	F	1.8 mg/kg, OD × 10	S–O
	19	F	2.0 mg/kg, OD × 10	P
	22	F	2.0 mg/kg, OD × 10	S–O
	26	F	2.2 mg/kg, OD × 10	S–O
	27	F	2.4 mg/kg, OD × 10	S–O
Cancer of the colon	8	F	1.0 mg/kg, OD × 15	O
	16	M	1.8 mg/kg, OD × 10	P
	20	F	2.0 mg/kg, OD × 10	NC
	23	F	2.0 mg/kg, OD × 10	NC
	25	M	2.2 mg/kg, OD × 10	NC
	30	F	1.2 mg/kg, OD × 11	S–O
Melanoma	2	F	0.5 mg/kg, OD × 15	S–O
	5	M	1.2 mg/kg, OD × 10	S–O
	13	F	1.6 mg/kg, OD × 10	NC
	24	M	2.0 mg/kg, OD × 10	NC
	28	F	0.55 mg/kg, OD × 10	P
Cancer of the lung	3	M	0.75 mg/kg, OD × 11	P
	17	M	2.0 mg/kg, OD × 10	P
	18	M	2.0 mg/kg, OD × 10	P
Soft tissue sarcomas	12	M	1.6 mg/kg, OD × 10	P
	29	F	1.2 mg/kg, OD × 10	P
Cancer of the ovary	14	F	1.8 mg/kg, OD × 10	S–O
Cancer of the pancreas	21	F	2.0 mg/kg, OD × 10	S
Lymphoma	11	F	1.6 mg/kg, OD × 10	S–O

*OD = daily dose.

†P = progression of disease; S = subjective remission; O = objective remission; NC = no change.

‡Criteria for objective remission: (*a*) decrease of 50% or more in the product of the two perpendicular diameters of the lesions being measured (two consecutive measurements); (*b*) no deterioration of the patient's general condition; (*c*) no evidence of new lesions.

yet known since the drug has been used for only 10 months; however, two patients treated early in the study (one with carcinoma of the breast and one with melanoma) have remained in remission for 6 months.

Both of the long term responses have occurred in patients receiving maintenance therapy, the patient with breast cancer receiving an average dose of 150 mg of 5-azacytidine per week, and the patient with melanoma receiving an average dose of 225 mg per week. The initial remissions in patients not receiving maintenance treatment were almost invariably short, lasting an average of 6 weeks; we did not see any remissions lasting longer than 12 weeks in these patients. It was possible, however, to re-induce remissions in two patients with breast cancer who had relapses, one 8 weeks and the other 10 weeks after the cessation of therapy.

An attempt was made to establish a maintenance regimen for the patients who had had remissions. The minimum total weekly dose that produced moderate but tolerable hematologic toxicity was usually 2.4 mg/kg given twice weekly. This dose usually caused leukopenia with a total wbc of 2500–4000 cells/mm^3 or thrombocytopenia with a platelet count of 80,000–110,000 cells/mm^3 or both. There was significant variation, however, in tolerance to the drug of patients receiving maintenance therapy. The maintenance dose was commonly associated with moderate nausea; however, no patient refused to take the medication because of this.

DISCUSSION

5-Azacytidine, a pyrimidine analog, has been found to be quite active in a small group of patients, most of whom have breast cancer or melanoma. The data gathered to date present several interesting points.

Remission appears to be at least partially independent of dose in that remissions occurred at dose levels that gave no significant toxicity. Also, remissions commonly occurred early in the treatment course. Three patients with breast cancer had obvious shrinkage of their tumors after 3 and 4 days of drug therapy and long before hematologic effects were noted, and a similar finding was noted in two of the patients with melanoma who had remissions.

It is apparent from the data obtained in animal studies that the total dose required to cause toxicity is significantly less when the drug is given intermittently over a prolonged period of time (ie, over 9 days) than when it is given as a short course of therapy.[9] It is our impression that this may be so for man also since the total dose required to cause significant toxicity in man was 16 mg/kg when the drug was given over a 10-day course and approximately 10 mg/kg when given over a 15-day course.

Since the primary problem with the administration of drug, as far as the patient is concerned, is nausea, and since the severity of the nausea and vomiting appears to be directly related to the amount of drug given per injection, it is our feeling that patient tolerance will be much greater when the initial loading course is given over a prolonged period of time. Fortunately, this type of regimen appears to be the most effective in animals as far as antitumor activity is concerned, and our data to date, although extremely sketchy, lead us to believe that this will be the case for man as well.

It is also apparent that, compared with both dogs and rodents, man can tolerate high doses of the drug even when the doses are calculated on a milligram-per-kilogram basis. The reason for this is not clear since there are no obvious differences in overall patterns of excretion (8,9). The relative activities of kinases, deaminases, and other pertinent enzymes in various species are not known, however, and drug incorporation would be related to these. Also, it seems that the mechanism of action of the drug in man is not related to that of 5-FU or cytosine arabinoside and that resistance to one of these agents is not correlated with resistance to 5-azacytidine.

Initial observations in these patients appear to indicate that 5-azacytidine is promising as an anticancer agent in the treatment of breast cancer and melanoma. The series is obviously too small to enable us to make decisions concerning the drug's efficacy in other tumors.

[9] *See* footnote 7.

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Phase II Study of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; NSC-79037) in the Treatment of Brain Tumors [1,2,3]

Derek Fewer, Charles B. Wilson, Edwin B. Boldrey, and Jean K. Enot [4]

SUMMARY

Thirty-eight patients with symptomatic recurrent primary or metastatic brain tumors were treated with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) at an initial dose of 120–130 mg/m² with subsequent doses determined by the degree of delayed toxicity. The interval between treatments was usually 6 weeks. Two groups of patients were treated: those who had received no prior chemotherapy and those who had previously received BCNU. The response rates were 33% and 0 respectively. The major dose-limiting toxic effect was delayed depression of the white blood cell and platelet counts; this depression was not cumulative with the schedule used. Significant toxicity was encountered more frequently in the patients who had received BCNU previously. We concluded (*a*) that CCNU was effective against recurrent brain tumors although the results were not any better than those attainable with BCNU in a similar group of patients, and (*b*) that cross resistance to CCNU occurred in patients treated previously with BCNU.

[Cancer Chemother Rep 56:421–427, 1972]

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) is one of a number of nitrosourea derivatives synthesized at the Southern Research Institute as potential anticancer agents (1). Interest in these compounds was aroused by the discovery of the considerable antitumor activity of 1-methyl-1-nitrosourea (NSC-23909) against L1210 leukemia implanted in mice both intraperitoneally (ip) and, more important, intracerebrally (ic) (2). At about the same time, Rall and Zubrod, in a review of the pharmacologic principles of drug therapy in the central nervous system, described the criteria which, ideally, must be met by a plasma agent crossing the blood-brain barrier by passive diffusion: the agent should be un-ionized at physiologic pH, it should be highly lipid-soluble, and it should not be bound to plasma proteins (3). It has been shown that the nitrosoureas fulfill these requirements (1).

The first of the nitrosoureas to be tested extensively in animals and man was 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962). Using BCNU labeled with radioactive carbon (^{14}C), DeVita et al showed that the breakdown products equilibrate rapidly between the cerebrospinal fluid (CSF) and plas-

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[3] CCNU was supplied by the Cancer Therapy Evaluation Branch, Chemotherapy, National Cancer Institute (NCI).

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ma (4). At optimal doses BCNU has considerably increased the lifespan of mice with ic implanted L1210 leukemia (1) and carcinogen-induced ependymoblastoma (5) and rats with carcinogen-induced glioma.[4] Phase II studies in patients with malignant brain tumors have shown BCNU to be superior to any previously tested drug in effecting clinical improvement not related to concomitant corticosteroid therapy (6,7).

Because of structural differences, CCNU is more lipid-soluble than BCNU (8). Distribution studies with radioactive CCNU in mice have shown that CCNU readily enters normal mouse brain tissue and mouse brain tumor and that a plasma-to-brain steady state ratio is reached soon after administration of the drug (9). In dogs given CCNU labeled with ^{14}C, levels of radioactivity in the CSF were two to three times greater than levels in the plasma during a 6-hour interval after injection (10).

CCNU was chosen for clinical trials because of its distinct life-prolonging effect in animals with ic implanted murine ependymoblastoma (9) and ic implanted L1210 leukemia (11). In a phase I study of CCNU (8), noticeable neurologic improvement occurred in three of three patients with glioblastoma; the dose-limiting toxic effect was a delayed, reversible depression of the platelet and leukocyte counts.

We are reporting the results of a phase II study of CCNU in a group of 38 patients with malignant brain tumors. The aims of this study were: (*a*) to test the efficacy of CCNU administered by the oral route in the treatment of primary and secondary malignant tumors of the neuroaxis; (*b*) to compare the results of CCNU therapy in patients previously untreated with chemotherapy with the results in patients in whom there was clinical deterioration after two or more courses of BCNU; and (*c*) to compare the number, quality, and length of responses obtained with CCNU with those obtained in a similar phase II study of BCNU.

PATIENTS AND METHODS

Between August 1970 and July 1971, 38 patients received CCNU one or more times. Patients eligible for acceptance into this study were: (*a*) those with primary malignant brain tumors who had had a partial tumor resection or biopsy, who had subsequently manifested clear clinical deterioration due to tumor regrowth, and who had tumors which could be measured by either brain scan or contrast radiographic studies; and (*b*) patients with intracerebral metastatic disease confirmed by removal or biopsy, or patients with intracerebral metastatic disease or brain stem gliomas not confirmed surgically but indicated on the basis of clinical examination and radiographic studies. Evidence of clinical deterioration and abnormal results of either brain scan, or radiographic studies, or both, were also required for inclusion of these patients in the study.

Patients were included in the study whether or not they had received a full or partial course of radiation therapy. If a full course had been given, it must have been completed 3 months before CCNU treatment was started. Similarly, prior chemotherapy with drugs other than BCNU was not a factor for exclusion from the study provided it had been completed at least 6 weeks before CCNU treatment was begun. When the diagnosis had been made at the time of resection or biopsy, the microscopic slides were reviewed for confirmation of tumor type.

We assumed that corticosteroids (eg, dexamethasone) have no direct antitumor effect, improving neurologic signs solely by their ability to reduce the cerebral edema associated with the neoplasm. Changes in steroid therapy were taken into consideration in the interpretation of changes in a patient's clinical condition, brain scans, and radiographic studies. We assumed as well that the standard anticonvulsant medications (ie, diphenylhydantoin sodium [Dilantin], phenobarbital, primaclone [Mysoline]), which the majority of our patients were taking, have no antitumor effect.

Patients were excluded from the study after acceptance if (*a*) death occurred within 4 weeks of the initial treatment or (*b*) another form of treatment was considered necessary for the better care of the patient during this same initial 4-week period.

[4] Gurcay, O., Wilson, C. B., Barker, M., et al. The effect of 1,3-bis(2-chloroethyl)-1-nitrosourea on a transplantable rat glioma. Unpublished data.

Case Evaluation

The initial inpatient clinical evaluation included a general physical and neurologic examination, urinalysis, complete blood cell count (cbc) and platelet count, and serum biochemical survey including determination of blood urea nitrogen (BUN), creatinine, glucose, serum glutamic oxaloacetic transaminase (SGOT), lactic dehydrogenase (LDH), alkaline phosphatase, and bilirubin levels. For each patient, a technetium-99M pertechnetate brain scan and one or more radiographic contrast studies pertinent to the type and location of the tumor were done. CSF was obtained for cytologic study and protein and sugar determination in some cases, and psychologic assessment was done when it was thought that evaluation of psychologic abnormalities might provide another means of following a patient's clinical course.

When a patient was re-admitted for retreatment, the evaluation was repeated except that the radiographic studies were repeated only if changes in the brain scan did not correlate well with the clinical changes (12) and there was a possibility of re-operation, or if the brain scan was not a useful test for the type or location of the patient's tumor.

After each treatment, each patient was discharged to his home, a nursing home, or another hospital, depending on his condition. Between treatments, the results of twice-weekly cbc and platelet counts were forwarded to the chemotherapy nurse who recorded them on a flow chart. No interim neurologic assessment was attempted.

Treatment

CCNU is supplied in 40- and 100-mg capsules which must be stored at temperatures below 0 C. The drug is administered by the oral route. With only two exceptions, patients were hospitalized before being given both the initial and the subsequent doses. The interval between treatments was 6 weeks with the exception that, near the end of the study, three patients received two doses each 1 month apart. The initial dose was 120–130 mg/m^2; subsequent doses were determined by the degree of delayed bone marrow depression.

Acute gastrointestinal toxicity, manifested by a single episode of nausea and vomiting, occurred in virtually all patients 3–4 hours after each treatment and, although often palliated, was never prevented by the commonly used antiemetics. To lessen the morbidity and, perhaps, to allow greater gastrointestinal absorption, a treatment schedule was designed whereby a patient would eat a normal breakfast and then would not receive anything by mouth until approximately 1:00 PM when he would be given the CCNU. In most instances, the patient would become ill later in the afternoon but subsequently would feel well enough to eat a normal supper.

End Points

A patient was considered to have failed to respond to CCNU if either no improvement or clinical deterioration was apparent at the time he was admitted for his third treatment (or for subsequent treatments). A minimum of two treatment intervals was necessary before we could make this decision because two patients in our BCNU series and one patient in the present CCNU series showed clear clinical deterioration at the time of the second treatment but improved subsequently. If a patient died as a result of the progression of his tumor before this time, but after 1 month had elapsed from the time of treatment, he was considered to be a nonresponder also.

At the present time, we have no end point for patients who continue to respond to CCNU (unless progressive bone marrow depression becomes chronic), and we plan to continue treatment for a minimum period of 12 months as long as the patients do respond.

We designated as responders those patients who showed, clear, clinical neurologic improvement unrelated to changes in corticosteroid therapy and those initially corticosteroid-dependent patients (with or without clinical improvement) from whom we were able to withdraw corticosteroids.

RESULTS

A total of 38 patients received CCNU. For nine patients, response to treatment was not

considered evaluable: for six, followup was inadequate; for two, findings at necropsy convinced us that the neurologic signs which had been under observation were unrelated to their tumors; and the ninth had what we considered to be irreversible brain damage due to the progression of tumor 2 weeks after the initial treatment. The remaining 29 patients were divided into two groups for evaluation of response; one group consisted of those patients who had received no prior chemotherapy (21 patients) and the other group consisted of those who had previously received BCNU (eight patients).

Table 1 shows the diagnoses of these 29 patients, the corresponding number of responses, and the duration of response (in months). The overall response rate (as well as that for glioblastoma alone) in those patients who had not received previous chemotherapy was 33%.

The 38 patients received a total of 83 doses of CCNU. Data from 27 of these treatment intervals were inadequate for evaluation of hematologic toxicity. We defined significant toxicity as a peripheral blood platelet count less than 30,000 cells/mm^3 or a peripheral white blood cell count (wbc) less than 2000 cells/mm^3.

In only four posttreatment periods did the platelet count remain normal; in 33, the count did not fall to less than 100,000 cells/mm^3, and in 17 it fell to between 30,000 and 100,000 cells/mm^3. In the majority of cases this occurred 3–5 weeks after treatment and persisted for 3–7 days. Significant platelet toxicity was noted in only two of 56 treatment intervals. This occurred during the same period as the usual depression in platelet count but tended to last longer, with 1–3 weeks elapsing before recovery to pretreatment values began. In no case was there clinically evident hemorrhagic diathesis. There was a higher incidence of significant toxicity in patients previously treated with BCNU than in patients who had had no prior chemotherapy. This presumably was related to the cumulative marrow toxicity of BCNU since, in all cases, BCNU treatment had been stopped only 6–8 weeks prior to the start of CCNU therapy.

No discernible drop in the wbc from the range of normal for each patient could be detected in 40 of 54 treatment intervals. A significant drop, as previously defined, occurred in 14 of 54 intervals, and usually this occurred 1–2 weeks later than the drop in platelet count with which it was always associated. The wbc

TABLE 1.—Chemotherapy data for 29 patients treated with CCNU

Diagnosis	Total No. of patients	Response fraction	Duration of response* (months)
Patients treated previously with BCNU:			
Glioblastoma	4	0/4	—
Brain stem glioma	2	0/2	—
Cord astrocytoma	1	0/1	—
Microglioma	1	0/1	—
	Total responses	0/8	
Patients not treated previously with antitumor drugs:			
Glioblastoma	15	5/15	1, 5, 6, 6, 7
Carcinoma of the lung	4	1/4	1
Medulloblastoma	1	0/1	—
Metastatic adenocarcinoma	1	1/1	3
	Total responses	7/21	

*Numbers underlined indicate the number of patients alive and still receiving treatment.

returned to normal in 1–3 weeks. No clinically evident infections occurred during these periods of granulocytopenia. Of seven patients who received three or more treatments at 6-week intervals, only one had a pattern of hematologic values consistent with cumulative toxicity, and this was mild. As a consequence, none of our patients received less than 85% of the starting dose while in this study. Of the three patients who received more than one treatment at 4-week intervals, two had hematologic values consistent with cumulative toxicity.

In the majority of patients we noted a two-fold to threefold rise in serum alkaline phosphatase level; only occasionally was there an associated change in serum LDH or SGOT levels. These values showed no tendency to rise further after repeated treatment. No other forms of toxicity were observed.

When there were no concomitant changes in corticosteroid therapy in patients with supratentorial tumors, we correlated serial changes in the clinical status with those of brain scans done at the corresponding times. There was a positive correlation (ie, the patient's condition and the scan results improved, the patient's condition and the scan results worsened, or the patient's condition and the scan results remained unchanged) in 29 of 33 intervals.

DISCUSSION

In a recent review of our own experience in a phase II study of BCNU treatment of brain tumors, we reported an overall response rate of 48% (12). These patients had been selected and their cases evaluated as in the present study. Since the pharmacologic and preliminary animal study data suggested that CCNU might be a more effective drug, we selected it for the present phase II study. The results in previously untreated patients, as described in table 1, show that treatment with CCNU using the present schedule is no more effective than treatment with BCNU. This conclusion is strengthened by our knowledge that three of four patients whose cases were not evaluable for response (table 1) underwent progressive deterioration and died within 1–3 months after treatment. These patients were excluded from the study because we had no opportunity to examine them after the initial treatment and thereby perhaps exclude another cause for their deterioration. If these patients had been included, the response rate would have been even lower.

The reasons for this less-than-expected response rate are not clear. Early in the study we feared that oral administration might lead to uneven, subtotal drug absorption; our concern was heightened by the almost universal occurrence of vomiting 3 hours after administration of CCNU. However, the data from animal studies on drug effect after oral administration (11), and the uniformity of drug toxicity in the phase I study (8) and in our present series tend to negate this possibility. It is possible that the present dose schedule is not optimal but, to date, the data from studies in animals (11) and man (8) indicate that the schedule used achieves the greatest antitumor effect with the least toxicity. Our own data seem to suggest that shortening the treatment interval from 6 to 4 weeks will lead to dose-limiting cumulative toxicity.

The lack of response in any of the patients who had previously received BCNU probably indicates that cross resistance to CCNU occurs in these patients. Since the two drugs are similar in structure and are thought to have identical modes of action (11), this is not surprising. Another possible interpretation of these data is that these patients, having failed to respond over a period of time to BCNU, were that much further along in their disease course before starting treatment with CCNU and therefore had less chance of responding. In none of these patients, however, was the neurologic condition any worse than that of a number of patients who had not previously received BCNU and who did respond to CCNU.

The two patients with metastatic tumors who responded to CCNU are interesting in that during the period of distinct improvement in the results of both the clinical neurologic examination and the brain scans, there was concomitant progression of systemic disease and, in fact, neither patient died as a result of the cerebral lesions. This phenomenon of a varied response by the same tumor in different locations has been observed in animals (13).

At the present time we have no answer to the questions of whether or when to terminate therapy in patients who are responding and who have a stable neurologic deficit. In the present series this problem did not arise, but in our phase II BCNU series we stopped treatment in two patients. One had a frontal, low-grade astrocytoma and had responded to BCNU for over 1½ years after initial recurrence of tumor. We stopped treatment because he had a persistant severe anemia that required multiple transfusions. Prior to cessation of therapy, his neurologic examination and brain scan had remained stable for over 7 months. Within 3 months after cessation of therapy, however, we observed a progressive deterioration in the clinical course of this patient; brain scans also indicated deterioration. The other patient had a recurrent posterior fossa ependymoma which had responded to BCNU treatment for over 20 months. He had had a minimal, stable neurologic deficit for 10 months and a normal brain scan for 4 months prior to cessation of BCNU therapy. At present, he has received no treatment for 5 months and shows no signs of deterioration.

The dose-limiting toxic effect of CCNU treatment was delayed depression of the peripheral wbc and platelet counts, similar to that observed in the phase I study (8). The toxicity to platelets was more universal in this study than in our previous phase II BCNU study. Also, in contrast to results observed in patients receiving BCNU (12), in only one patient receiving three or more courses of CCNU were there any signs of cumulative toxicity.

The very high positive correlation between brain scan and clinical course reinforces our earlier view that serial brain scans provide the most reliable measurement of tumor in relation to drug response. In the present study we did not repeat angiographic studies because angiographic changes in an earlier study had correlated poorly with clinical observations.

Although the degree of functional improvement was not a prime determinant in this study, we noted the disappearance of moderate degrees of dysphasia, hemiparesis, and dullness of sensibility in various patients.

One advantage of CCNU treatment is that the drug may be administered orally in a single dose. Although in the present study the majority of patients were admitted to the hospital for retreatment, we feel that ambulatory patients can have a brain scan, be examined, and receive their doses of CCNU in half a day without being hospitalized. For patients requiring hospitalization, this method of administration shortens the hospital stay. Although CCNU is clearly an effective agent against both primary and metastatic brain tumors, the present study suggests that treatment with CCNU results in a lower response rate than treatment with BCNU, and that a cross-resistance phenomenon exists between these two drugs. The rate of response to CCNU reported here is similar to that observed in a previously reported study (14). Although possibly less effective than BCNU when given alone, CCNU may prove to be a useful drug in combination chemotherapy for brain tumors and the drug deserves a trial in this context.

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